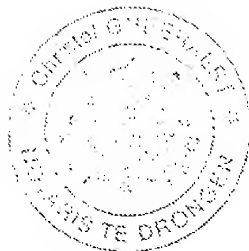


Opened <u>Dec 30</u> 20 <u>02</u>
Décapitée le <u>Dec 30</u> 20 <u>02</u>
<u>[Signature]</u>
Commissioner of Patents Commissaire des brevets
In presence of <u>[Signature]</u>
en présence de l'examinateur

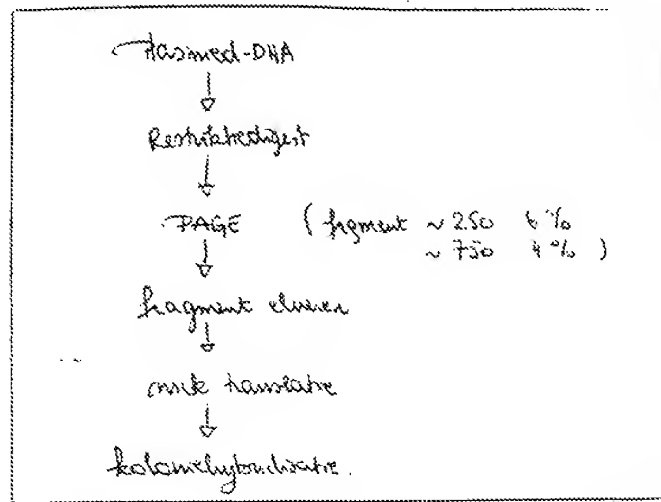


This is EXHIBIT FIERS-9
to
the Affidavit of Walter C. Fiers
sworn before me
this 19th day of November, 2001

Commissioner for Oath or Notary Public

KOLONIEHYBRIDISATIE

Schema



① ① 7672 (band 4) fragment ← G Volkeant.

mikrotranslatie

100 µl α³²P dATP oplossing (nieuw)
 15 µl 2x NT buffer
 10 µl DNA-fragment (7672, ± 0.5 µg)
 2.5 µl dLTP
 dGTP } 100 µM elk
 dTTP
 1.5 µl NT H₂O
 0.5 µl DNase I (0.1 ng/µl)

⇒

10 min 37°C
omw. op ijs

0.5 µl (0.5 µl)

+ 0.5 µl DNA polymerase I (5U/µl)
Boehringer
14 à 15°C

draaijes om 15 min
 (0.5 µl) 30 min
 60 min
 90 min
 120 min
 150 min

50 µl blaauw

→ nemen met 0.5 µl micro-vef
 spuiten op DEAE-12 papier → vullen in 0.5 M KH₂PO₄,
 0.5 M KH₂PO₄,
 H₂O
 0.5 M

Denhardt-solution { 0.02 % glycyl
 { 0.02 % polyvinylpyrrolidone
 { 0.02 % BSA

↓
 20x stock → sample mixtures

SET { 0.15 M NaCl
 { 0.03 M Tris, pH 8
 { 0.001 M EDTA

↓
 100x stock

5.2. fractie. 3. juli. y. 6.10
 dagen.
 Bellen in 70-100.0

(1 liter - min.
 15.000.000)

tot $\pm 70 \cdot 10^6$ tellen in een probe

Colony hybridization

1. Filter uit 3/3/17 preincuberen in 5x Denhardt
 in SET
 in pefylaat, niet schuddeend

DN 68°C
 2 hr

2. Rinse 5x SET
 1x Denhardt
 0.5% SDS

15 min 68°C

(SDS zit toevoegen voor Denhardt solution - 1x)

in pefylaat, niet schuddeend

3. x 20 x 20 plaat

→ filter na afschrijven exp. beppen
 doornijf eenheden
 tweed. glasplaat exp. en bepalen.

* PROBE DENATUREREN (10.000 → 10⁶ tellen) 5 min 100°C

→ Na G-50 Sybraden

toevoegen aan 7 à 10 ml Rinse-oplossing

→ opzetten in spuit → in glasplaat druppelen

hybridisatie

DN 68°C
 (2x langer)

4. 1^{ste} WAS 25 ml 2x SET
 0.5% SDS
 2^{de} WAS 25 ml

in pefylaat

4 hr 68°C
 2 hr 68°C

niet schuddeend

5. wassen in 3x 10 min
overvloedig

DN 68°C

200 à 300 ml

in handen
 (1 ml tel van 1 liter 5.000.000)

pueren (20 ml) afgevuurd → keuze op basis van kolom

gekozen kolom {

- * positief
 - $\pm 9,10$
 - C_9
 - L_2
 - kolom $1/4 : C_4$
 - $2/12 : C_5$
- * negatief
 - C_8
 - C_4
- * twijfelachtig
 - C_4
 - $g 8,9$

11/1

Gesolgende procedure

- cellen afcentrifugeren 5000 gpm 10 min Sm 24.
heruspendelen in 5 ml TES. warmen
5000 gpm 10 min Sm 24
- heruspendelen in 1 ml | 50 mM Tris pH 8
10% sikraal
- + 0,1 ml 10 mg/ml lysozyme in 25 mM Tris pH 8
5 min kamertemperatuur (20 min op ijs)
- + 0,1 ml 25 mM EDTA pH 8
- + 1 ml TLM 1 0,2% TX 100
50 mM Tris pH 8
25 mM EDTA
- zacht mengen
15 min kamertemperatuur (wt langer)
- 30 min 17.000 gpm SS34 - clearing spin
- SN + 3 ml phenol (equl) goed schudden
30 min 5000 gpm SS34
- 2x etherextractie → verwijderen van TX100 (wt later misvat hebben met digeren)
: dus vloeistof.
- + 1 vol isopropanol → -20°C
- 30 min 464 3000 gpm - pellets drogen
heruspendelen in 1/100 STE. loep

2/12 AS blüht jetzt
4/4 C1 ersten späten anfang
(1) rezeption von kaliumhydroxide

5

11/1/82

Sau 3A digest van $\left\{ \begin{array}{l} \text{BR 322 (4 } \mu\text{g)} \\ \text{STAN-1 (4 } \mu\text{g)} \end{array} \right.$

$\left\{ \begin{array}{l} \text{f-2} \\ \text{f-3} \\ \text{h-2} \end{array} \right.$

mengsel van telkens 20 μL met telkens 4 μL 10x TBE, 5 μL DNA

dit materiaal wordt
hyperficeerd
Het is immers uit onder
aan dat het materiaal
of 22 mg maal van
(1x100) Sau 3A eruit
hebben \rightarrow het inhiberen
of restische digestie.

30 μL DNA-mengsel
3,5 μL 10x buffer
2 μL Sau 3A I

35,5 μL

2 hr 37°C

+ 1 μL (Rho A (0,5 $\mu\text{g}/\text{L}$))

20 min 37°C

+ 3 μL Maat 10x 217
+ 80 μL EtOH

\rightarrow -20°C

\rightarrow afblazen
op een gel: 1,7%

Verdere aanwijzing in Sau 3A 28K-fragment : 4,5% TBE - PAGE. 27

Band uitnemen
DNA elueren

\sim 100 000 kellen (50 ml (concentr))

\downarrow 1M NaCl

koloniekultuur tot f5-f8

10x 50 $\mu\text{g}/\text{ml}$ calf thymus extracted DNA

\rightarrow Zeer goed resultaat! 28

Konstruktion von neuen probes

⑤

26K-Son3A-Fragment

Zie hervor
Digest
2x 4,5% PAGE (TEB) (2x 30µg).

⑥

IF-8 - Hinf I - Fragment

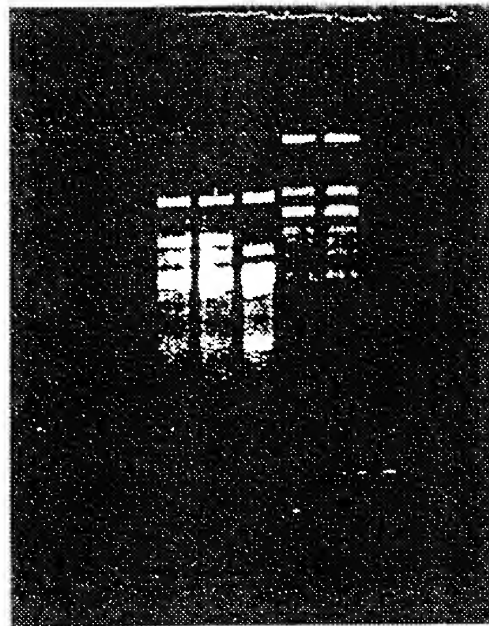
2x
20 µl DNA (~30µg)
65 µl 5x H₂O
10 µl 10x glyco
25 µl Hinf I (200 U/ml)
100 µl total

IF-5 - Hinf I - Fragment Hsp I

20 µl DNA (~30µg)
60 µl 5x H₂O
10 µl 10x glyco
25 µl Hinf I (200 U/ml)
5 µl Hsp I (200 U/ml)
100 µl

4 uur 37°C

→ 2,2% agarose-gel (inhalten of digest volledig is)



~~~~~  
Hinf I      Hsp I

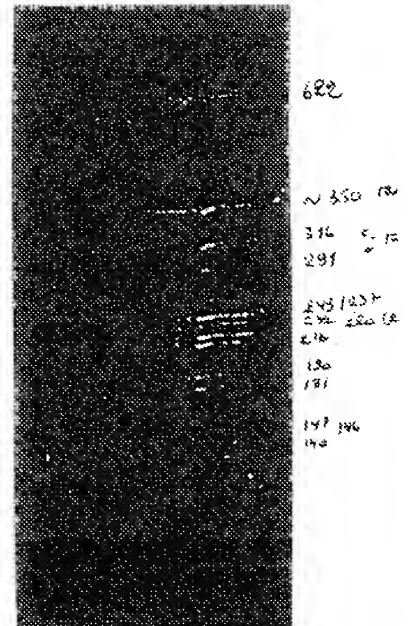
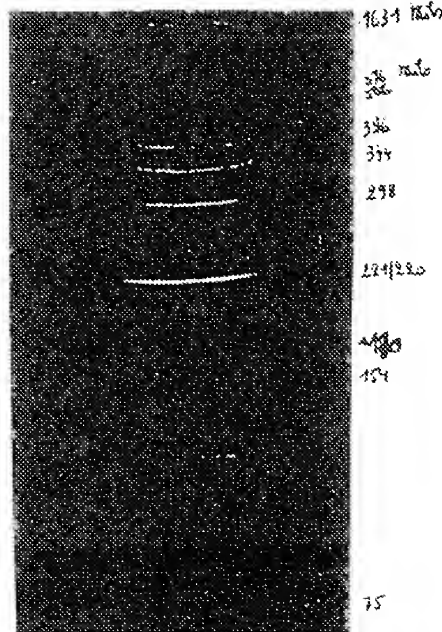


↳ preparatieve gel

a)

IF-8 : 6% PAGE (TEB)  
250 V overnight

IF-5 : 4.5% PAGE (TEB)  
250 V overnight



elute in 1M NaCl  
preplate met 0.2M NaCl pH 5.1  
1 vol digenase

heropgenomen in IF-8 1/10 STE  
IF-5 : 1x afwas → het fragment met de linkerarm loopt  
samen met het interne fragment.

koppelen met Hha I ~40 maal v.h.  
fragment met tails afpakken  
→ ginds

21.5 µl DNA IF-5  
2.5 µl 10x buffer  
1 µl Hha I. (1200 U/ml)  
2 hr 37°C.

Inchougen in sucrose denaturator.

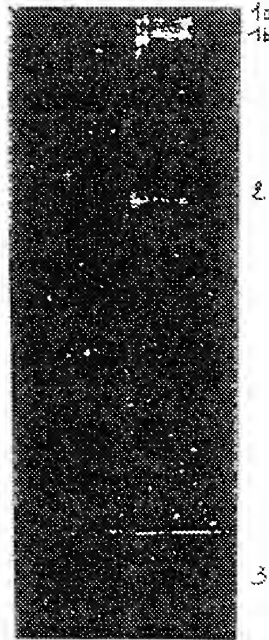
2.2% agarose -fortgel



digest met ck  
(molecular weight 7)

g) beide Seiten of 6% PAGE (TEB)  
IF-8 IF-5

(sammelt 250V)



→ wochl. deutlich overlapp.  
wenigste

banden untersten  
elute mit 1M NaCl

4x IAA extrakt  
1x ether extrakt  
EtOH precipitieren



IF-8 = interferon-spezifisch

Onk-translates von IF-8 HmP - fragment

① 50  $\mu$ l  $\alpha^{32}$ P-DATP (2.1000 mCi/ml)  
(1 ml / ml) — 25 pmol '80  
15  $\mu$ l 2x NT buffer  
5  $\mu$ l DNA - gelung  
25  $\mu$ l dGTP  
CTP  
TTP  
7  $\mu$ l et H<sub>2</sub>O  
0.5  $\mu$ l  $\mu$ l (E ul)  
30  $\mu$ l

Strahlendose mCi : 0.12  
- 1.2  
- 30

- skolnane na 0  
15  
6  
47 min

Syladex C<sub>7</sub>-50

① mRNA translation  $\alpha^{32}\text{P}$  AAT 29

201 good  
resulted

Kolonchubudates

- ↳ Kontrolle ob  $f_{-11}$  = positiv

Kleef op d. 7 = zeer jonkhef

37

Background zeer laag!

- (2) • verk. hand. v.  $10\frac{1}{2}$  - 7 flm. f fragment v. verk. hand. v.  $10\frac{1}{2}$  - 7 flm. f  
b/  $10\frac{1}{2}$  - 7 flm. f v. verk. hand  
→ materiaal is te oud (1,5 maand.)

- ③ - mak. hand. ④ - 50 000 fellen (Grenzkas.)  
 -  $Q_1$  - feller (= FS | 18-14)  
 -  $Q_2$  - feller (= 11)

↳  $19: y(1)$  muss allzeit mit  $x(1)$  übereinstimmen.



## Klonaanalyse van G<sub>1</sub>-d7

20 ml LB/tet cultuur ON opgeven

lyseprocedure v. H1180

na ~~de~~ EtOH precipitatie: materiaal heropnemen in 200 µl st H<sub>2</sub>O  
(geen EDTA)

• RNAse digest: + 10 µl Pancreas RNase (0.5 mg)  
20 min 37°C

• fenolise, etherextractie

• + 50 µl LAAC 0.2 M in st H<sub>2</sub>O → 5 mm  $\bar{y}$

afcentrifugeren in Epp centrifuge

SNB nemen  
met drogen!

• heropnemen in 250 µl 10 mM EDTA pH 8  
(jellek lost niet zo gemakkelijk op)

• + 25 µl D.E.N.T.A.T.E pH 5.1  
• 500 µl EtOH

Agarose - elektroforese

|                    | DNA op       | scaphus | st H <sub>2</sub> O | enzym |        |
|--------------------|--------------|---------|---------------------|-------|--------|
| IF-8               | 1 (~15 µg)   | 2       | 16                  | 1     | Hin I  |
| IF-8               | 1 (~15 µg)   | 2       | 16                  | 1     | Hin I  |
| IF-8               | 1 (~15 µg)   | 2       | 16                  | 1     | Sma 31 |
| G <sub>1</sub> -d7 | 5            | 2       | 11                  | 2     | Hin I  |
| G <sub>1</sub> -d7 | 5            | 2       | 11                  | 2     | Hin I  |
| G <sub>1</sub> -d7 | 5            | 2       | 11                  | 2     | Sma 31 |
| pH <sub>2</sub> -7 | 1.5 (~17 µg) | 2       | 15.5                | 1     | Hin I  |



3 uur 37°C  
mengen in Sorant tot ± 10 µl



op 2.2% agarosegel (EB).

1 mm ribb  
gevoet in rubber  
clut 50 V tot een, dan 100 V



overige materiaal naar Guido Volckert.

# koloniekultures (vervolg)

## gekoren kloons

G<sub>1</sub>d<sub>7</sub>

kloon n°2

1'11 d1

n°3

1'11 f6

n°4

1'11 g2

n°5

1'13 a12

n°6

1'14 a9

n°1

1'15 e6

n°7

1'15 g2

n°8

1'18 b10

n°9

1'18 e1

n°10

1'19 g5

n°11

1'113 f12

n°12

2'11 b6

n°13

2'12 a3

n°14

2'13 f10

n°15

2'14 g11

n°16

2'17 b5

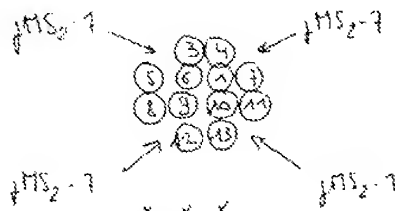
n°17

2'17 c6

n°18

Alle gekoren kloons opname op filter geest : VW-1

32

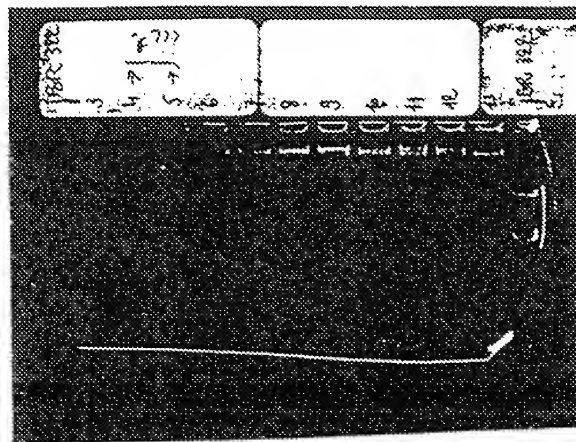


non denat. DNA van  
 5' 100°C " " G<sub>1</sub>d<sub>7</sub> (telken = 1 µg)  
 5' 100°C " " kloon 2

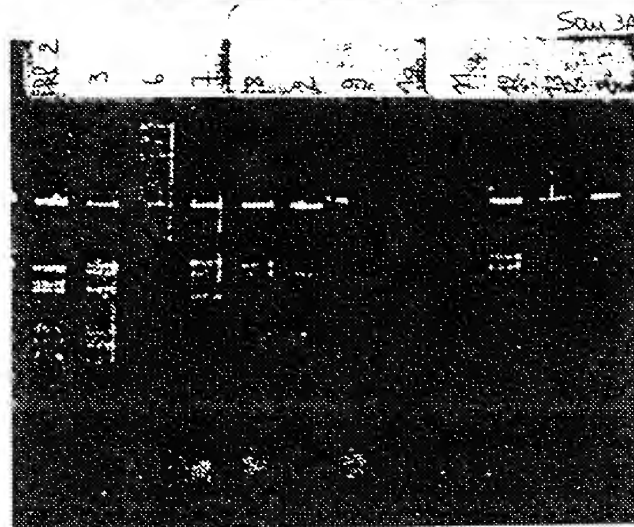


chrom. analysis - restriction analysis - electrophoresis

20 ml LB/let kultures  
chloramphenicol - ampicillin  
Triton-lyse  
↓  
1% agarose gel



Restriktionsenzyme





telken 2,2% agarose en/of 5,5% acrylamide

Alle enzymatische reacties gebeurd in 30  $\mu$ l bij 37°C in 1x opko

10  $\mu$ l + 3  $\mu$ l BFB  $\rightarrow$  22% agarose  
(wtz. 1,5%)  
2  $\mu$ l + 6  $\mu$ l BFB  $\rightarrow$  55% acrylamide

### typisches

- 2  $\mu$ l poly(v)-glycerol (G. Volekaut)

+ 20  $\mu$ l poly(v)-degred. buffer

NaHCO<sub>3</sub> / Na<sub>2</sub>CO<sub>3</sub> 50 mM pH 9  
Edta 1 mM



in koelkast 2 hr 30°C



in Eppendorf

+ 20  $\mu$ l 1M NaAc pH 5.1  
+ 20  $\mu$ l EtOH.



- 50  $\mu$ l  $\gamma$ ATP droegdragen in Eppendorf



benzylamine in 20  $\mu$ l 1x kinase buffer

+ 0.5  $\mu$ l kinase, 25 min 37°C

- G-50 Sephadex

Kinase van poly(v)

G-50 Sephadex

|   |     |
|---|-----|
| 1 | 1.0 |
| 2 | 1.0 |
| 3 | 1.0 |
| 4 | 1.0 |
| 5 | 1.0 |
| 6 | 1.0 |
| 7 | 1.0 |
| 8 | 1.0 |

- Gel (agarose of acrylamide)
  - + ~ 250 ~~ml~~ kellen → NIET OP het gel spotten!
  - + 25 ml trisacetate buffer (G Valkeest).
  - 1/2 hr schudden, kamet.
  - spelen met trisacetate buffer (ex 5 min)
  - antibiotagruppen

33

34

35

# Diagrams

① Cl 2 Alu I.  
Alu I / Hsp I.  
Hsp I.  
Rsa I.  
Ara II  
Cl 1 Rsa I  
Ara II.  
pBR-ref.

agarose

② id. acrylamide

③ Cl 2 Hinf I / Hcl II.  
Hinf II  
Hinf I.  
Hsp I / Hcl I.  
Pvu I / Bst NI.  
Pvu I / Hae III.  
pBR-ref.

agarose

④ id. acrylamide

⑤ Cl 1. Eco RI.  
Sma 3A.  
Cl 3 Alu  
Sma 3A.  
Hsp I.  
Hinf I.  
Hinf - Hind I.  
Eco RI  
Hha I.  
pBR-ref.

agarose

⑥ Cl 3 id. acrylamide

⑦ Cl 3 Hinf.  
Hinf I.  
Scl I  
Sma 3A.  
Alu

agarose

⑧ id. acrylamide

⑨ Cl 3 Hd I.  
 Hd II - Alu I.  
 R I.  
 R II - Alu I.  
 Cl 6 Hd I.  
 Hd II - Alu I.  
 R I.  
 R II - Alu I.  
 Cl 7 Hd I.  
 Hd II - Alu I.  
 R I.  
 R II - Alu I.  
 per ref.

agarose

⑩ Cl 3 { Hd I.  
 6 { Hd II - Alu I.  
 7 { Alu I.

acrylamide

⑪ Cl 3 { R I.  
 6 { R II - Alu I.  
 7 {

acrylamide

⑫ Cl 6 Alu I.  
 Eco R I.  
 Hm I.  
 Sam 3A  
 per ref.

agarose

⑬ Cl 8 Alu I.  
 Eco R I.  
 Hm I.  
 Sam 3A  
 per ref.

agarose

⑭ Cl 6 { Alu I.  
 8 { Eco R I.  
 Hm I.  
 Sam 3A  
 per ref.

acrylamide

⑮ Cl 1 { Rsa I.  
 2 {  
 3 {  
 4 {  
 8 {

acrylamide

①① Cl 3  
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23) a  $\left. \begin{array}{c} 3 \\ 6 \\ 7 \\ 8 \\ 9 \end{array} \right\}$

Pvu I.

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24) a  $\left. \begin{array}{c} 3 \\ 6 \\ 7 \\ 8 \\ 9 \end{array} \right\}$   
a  $\left. \begin{array}{c} 11 \\ 7 \end{array} \right\}$

Hind II / Pvu I.

agarose

Aba I.

25) a  $\left. \begin{array}{c} 3 \\ 6 \\ 7 \\ 8 \\ 9 \end{array} \right\}$

Pvu I - Pvu I.

agarose

26) a  $\left. \begin{array}{c} 3 \\ 3 \\ 3 \\ 6 \\ 7 \\ 8 \\ 9 \end{array} \right\}$

Bgl I.  
Bgl II - Eco RI.

agarose

Pst I.

27) a  $\left. \begin{array}{c} 3 \\ 6 \\ 7 \\ 3 \\ 6 \\ 7 \\ 6 \\ 7 \\ 9 \\ 11 \\ 13 \end{array} \right\}$   
a  $\left. \begin{array}{c} 3 \\ 6 \\ 7 \\ 3 \\ 6 \\ 7 \\ 6 \\ 7 \\ 9 \\ 11 \\ 13 \end{array} \right\}$   
a  $\left. \begin{array}{c} 3 \\ 6 \\ 7 \\ 3 \\ 6 \\ 7 \\ 6 \\ 7 \\ 9 \\ 11 \\ 13 \end{array} \right\}$

Bgl I / Hda I.

agarose

Bgl II / Pvu I.

Hinf I / Pst I.

28) a  $\left. \begin{array}{c} 3 \\ 6 \\ 7 \\ 3 \\ 6 \\ 4 \\ 3 \\ 6 \\ 7 \\ 3 \\ 1 \end{array} \right\}$   
a  $\left. \begin{array}{c} 3 \\ 6 \\ 7 \\ 3 \\ 6 \\ 4 \\ 3 \\ 6 \\ 7 \\ 3 \\ 1 \end{array} \right\}$   
a  $\left. \begin{array}{c} 3 \\ 6 \\ 7 \\ 3 \\ 6 \\ 4 \\ 3 \\ 6 \\ 7 \\ 3 \\ 1 \end{array} \right\}$   
a  $\left. \begin{array}{c} 3 \\ 6 \\ 7 \\ 3 \\ 6 \\ 4 \\ 3 \\ 6 \\ 7 \\ 3 \\ 1 \end{array} \right\}$

Hae III / Hda I

agarose

Hae III / Bgl II

Hda II / Pvu II

Pvu I / Pvu II

(29) Cl 3 }  
           6 }  
           7 } Pst I / Hae III. agarose  
       Cl 3 }  
           6 }  
           7 } Pvu II / Hae III.

(30) Cl 3 }  
           6 }  
           7 } Pst I / Hae III. acylamide  
       Cl 3 }  
           6 }  
           7 } Pvu II / Hae III.  
       Cl 3 }  
           6 }  
           7 } Bgl II / Hae II.

(31) Cl 13 }  
       Cl 3 }  
           6 }  
           13. } Hda II / Bgl II. agarose 1.5%  
       pse up } Hda II / Bgl I. 1.8%

(32) Cl 3 }  
           6 }  
           7 } Bgl II. agarose.  
       Cl 3 }  
           6 }  
           7 } Bgl II / Pvu II.  
       Cl 3 }  
           6 }  
           7 } Bgl II / Hind II.  
       Cl 7 }  
           6 }  
           7 } Rsa I / Hind II.

(33) Cl 19 }  
           20 }  
           21 }  
       Cl 13 }  
       Cl 13 } Hae III. 3  
                   Hae III.  
                   Rsa I.

(34) Cl 6 } Hinf. agarose  
 7 }  
 21 }  
 Cl 21 Eco R II.  
 Cl 21 Taq I.

(35) Cl 19 } Pvu I - Pvu II. agarose  
 20 }  
 21 }  
 Cl 19 } Rsa I.  
 20 }  
 21 }  
 Cl 19 } Bgl II - Hind II.  
 20 }  
 21 }  
 Cl 19 } Hinf.  
 20 }  
 21 }  
 Cl 19 } Hsp I.  
 20 }  
 21 }

(36) Cl 19 Hinf. agarose  
 Hinf - Pvu II  
 Eco R II.  
 Taq I - Rsa.  
 Cl 20 Eco R II.  
 Taq I.  
 Alu I.  
 Cl 21 Alu I.  
 Cl 26K-1 { Rsa I.  
 Bst E II.  
 Mbo I - Rsa I.  
 Mbo II - Hinf.  
 Mbo II - Sau 3A

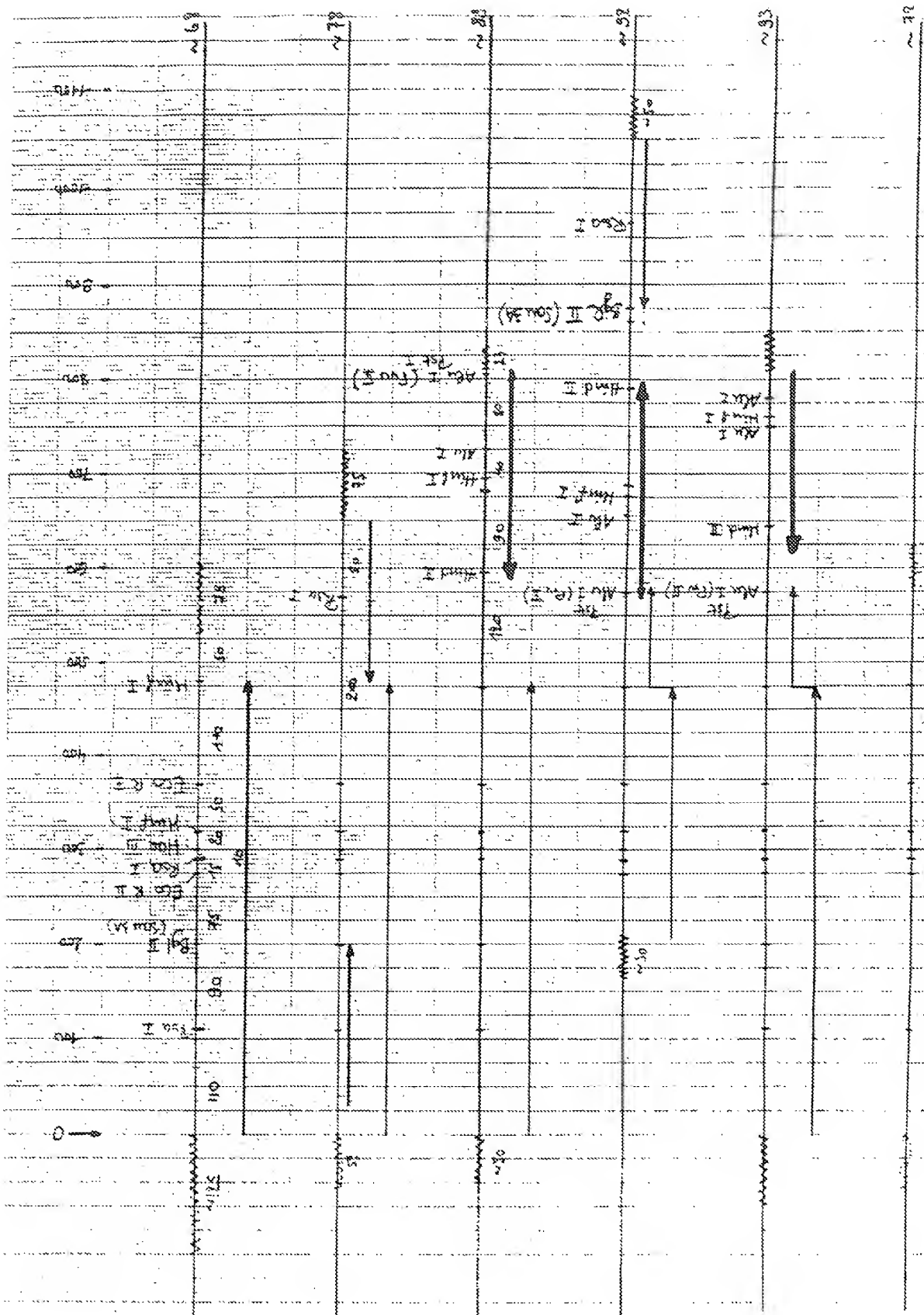
(37) Cl 19 Rsa - Taq I. agarose  
 Cl 20 Rsa - Taq I.  
 Cl 26K-1 Mbo II  
 Cl 26K-1 { Mbo II - Hinf.  
 Mbo II - Rsa I.  
 Mbo II - Sau 3A

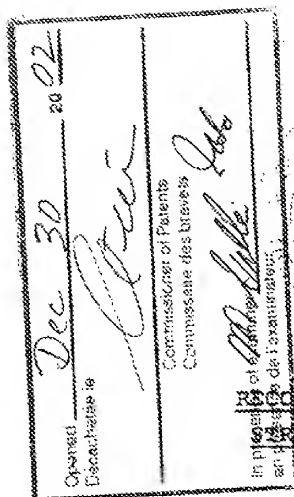


(38)

Cl 20  
Cl 19  
Cl 6

Tag I  
Tag I - Rsa  
Tag I - Rsa





This is EXHIBIT FIERS-10  
to  
the Affidavit of Walter C. Fiers  
sworn before me  
this 23<sup>rd</sup> day of November, 2001

FIERS  
88  
DRAFT

Commissioner for Oath or Notary Public

RECOMBINANT DNA MOLECULES AND THEIR USE IN PRODUCING  
STRUCTURAL GENES FOR HUMAN FIBROBLAST INTERFERON

TECHNICAL FIELD OF INVENTION

This invention relates to recombinant DNA molecules and their use in producing structural genes for human fibroblast interferon. The recombinant DNA molecules disclosed herein are characterized by DNA sequences that code for polypeptides whose amino acid sequence and composition are substantially consistent with human fibroblast interferon.

BACKGROUND ART

Two classes of interferons ("IF") are known to exist. Interferons of Class I are small, acid stable (glyco)-proteins that render cells resistant to viral infection (A. Isaacs and J. Lindenmann, "Virus Interference I. The Interferon", Proc. Royal Soc. Ser. B., 147, pp. 258-67 (1957) and W.E. Stewart, II, The Interferon System, Springer-Verlag (1979) (hereinafter "The Interferon System"). Class II IFs are acid labile. At present, they are poorly characterized. Although to some extent cell specific (The Interferon System, pp. 135-45), IFs are not virus specific. Instead, IFs protect cells against a wide spectrum of viruses.

Two antigenically distinct species of Class I human interferon ("HIF") are known to exhibit IF activity. One IF species, fibroblast interferon ("F IF"), is produced upon appropriate induction in diploid fibroblast cells. Another IF species, leukocyte interferon ("Le IF") is produced together with minor amounts of F IF upon appropriate induction in human leukocyte and lympho-

blastoid cells. Both are heterogeneous in regard to size, presumably because of the carbohydrate moiety. F IF has been extensively purified and characterized (E. Knight, Jr., "Interferon: Purification And Initial Characterization From Human Diploid Cells", Proc. Natl. Acad. Sci. USA, 73, pp. 520-23 (1976)). It is a glyco-protein of about 20,000 molecular weight (M. Wiranowska-Stewart et al., "Contributions Of Carbohydrate Moieties To The Physical And Biological Properties Of Human Leukocyte, Lymphoblastoid And Fibroblast Interferons", Abst. Ann. Meeting Amer. Soc. Microbiol., p. 246 (1978)).

✓ Its amino acid composition has been determined (E. Knight, Jr. et al., "Human Fibroblast Interferon: Amino Acid Analysis And Amino-Terminal Amino Acid Sequence", Science, 207, pp. 525-26 (1980)). Elucidation of its amino acid sequence is in progress. To date, the amino acid sequence of the NH<sub>2</sub> terminus of the mature protein has been reported for the first 13 amino acid residues: Met-Ser-Tyr-Asn-Leu-Leu-Gly-Phe-Leu-Gln-Arg-Ser-Ser... (E. Knight, Jr. et al., supra). Two distinct genes, one located on chromosome 2, the other on chromosome 5, have been reported to code for F IF (D.L. Slate and F.H. Ruddle, "Fibroblast Interferon In Man Is Coded By Two Loci On Separate Chromosomes", Cell, 16, pp. 171-80 (1979)).

✓ Le IF has likewise been purified and characterized. Two components have been described, one of 21000 to 22000 and the other of 15000 to 18000 molecular weight (K.C. Zoon, et al., "Purification And Partial Characterization Of Human Lymphoblastoid Interferon", Proc. Natl. Acad. Sci. USA, 76, pp. 5601-605 (1979)). A portion of the amino acid sequence of Le IF has also been determined, i.e., 20 amino acids from the amino terminus of the mature protein (K.C. Zoon et al., "Amino-Terminal Sequence Of The Major Component Of Human Lymphoblastoid Interferon", Science, 207, pp. 527-28 (1980)). A comparison of the initial amino acid sequence of F IF and Le IF reveals no detectable homology within the first 13 amino acids. The total amino acid compositions of the two species are also distinct. In addition, degradation of the sugar residues of the two species by periodate indicates that the carbohydrate structure of the two IFs is different (M. Wiranowska-Stewart et al., supra).

The two species of HIF have a number of different properties. For example, anti-human Le IF antibodies are less efficient

against F IF and anti-sera to human F IF have no activity against human Le IF (The Interferon System, p. 151) and Le IF displays a high degree of activity in cell cultures of bovine, feline or porcine origin whereas F IF is hardly active in those cells but has been reported to be active in rat cells (P. Duc-Goiran et al., "Studies On Virus-Induced Interferons Produced By the Human Amniotic Membrane And White Blood Cells", Arch. Gef. Virus Forsch., 34, pp. 232-43 (1971)). In addition, the two IFs result from different mRNA species (and therefore from presumable different structural genes) that code for polypeptides of different primary sequence (R.L. Cavalieri et al., "Synthesis of Human Interferon by Xenopus laevis Oocytes: Two Structural Genes for Interferon in Human Cells", Proc. Natl. Acad. Sci. USA, 74, pp. 3287-91 (1977)).

Although both Le and F IFs occur in a glycosylated form, removal of the carbohydrate moiety (P.J. Bridgen et al., "Human Lymphoblastoid Interferon", J. Biol. Chem., 252, pp. 6585-87 (1977)) or synthesis of IF in the presence of inhibitors which preclude glycosylation (W.E. Stewart, II et al., "Effect of Glycosylation Inhibitors On The Production And Properties Of Human Leukocyte Interferon", Virology, 97, pp. 473-76 (1979); J. Fujisawa et al., "Nonglycosylated Mouse L Cell Interferon Produced By The Action Of Tunicamycin", J. Biol. Chem., 253, pp. 8677-79 (1978); E.A. Havell et al., "Altered Molecular Species Of Human Interferon Produced In The Presence Of Inhibitors Of Glycosylation", J. Biol. Chem., 252, pp. 4425-27 (1977); The Interferon System, p. 181) yields a smaller form of IF which still retains most or all of its IF activity.

Both F IF and Le IF may, like many human proteins, be polymorphic. Therefore, cells of particular individuals may produce IF species within each of the more general F IF and Le IF classes which are physiologically similar but structurally slightly different than the <sup>protein</sup> class of which it is a part. Therefore, while the protein structure of the F IF or Le IF may be generally well-defined, particular individuals may produce IFs that are slight variations thereof.

IF is usually not detectable in normal or healthy cells (The Interferon System, pp. 55-57). Instead, the protein is produced as a result of the cell's exposure to an IF inducer. IF inducers are usually viruses but may also be non-viral in character,

such as natural or synthetic double-stranded RNA, intracellular microbes, microbial products and various chemical agents. Numerous attempts have been made to take advantage of these non-viral inducers to render human cells resistant to viral infection (S. Baron and F. Dianzani (eds.), Texas Reports On Biology And Medicine, 35 ("Texas Reports"), pp. 528-40 (1977)). These attempts have not been very successful. Instead, use of exogenous IF itself is now preferred.

As an antiviral agent, HIF has been used to treat the following: respiratory infections (Texas Reports, pp. 486-96); herpes simplex keratitis (Texas Reports, pp. 497-500; R. Sundmacher, "Exogenous Interferon in Eye Diseases", International Virology IV, The Hague, Abstract nr. W2/11, p. 99 (1978)); acute hemorrhagic conjunctivitis (Texas Reports, pp. 501-10); adenovirus keratoconjunctivitis (A. Romano et al., ISM Memo I-A8131 (October, 1979)); varicella zoster (Texas Reports, pp. 511-15); cytomegalovirus infection (Texas Reports, pp. 523-27); and hepatitis B (Texas Reports, pp. 516-22). See also The Interferon System, pp. 307-19. In these treatments F IF and Le IF may display different dose/response curves. However, large-scale use of IF as an antiviral agent requires larger amounts of HIF than heretofore have been available.

IF has other effects in addition to its antiviral action. For example, it antagonizes the effect of colony stimulating factor, inhibits the growth of hemopoietic colony-forming cells and interferes with the normal differentiation of granulocyte and macrophage precursors (Texas Reports, pp. 343-49). It also inhibits erythroid differentiation in DMSO-treated Friend leukemia cells (Texas Reports, pp. 420-28). Some cell lines may be considerably more sensitive to F IF than to Le IF in these regards (S. Einhorn and H. Strander, "Is Interferon Tissue-Specific? - Effect Of Human Leukocyte And Fibroblast Interferons On The Growth Of Lymphoblastoid And Osteosarcoma Cell Lines", J. Gen. Virol., 35, pp. 573-77 (1977); T. Kuwata et al., "Comparison Of The Suppression Of Cell And Virus Growth In Transformed Human Cells By Leukocyte And Fibroblast Interferon", J. Gen. Virol., 43, pp. 435-39 (1979)).

IF may also play a role in regulation of the immune response. For example, depending upon the dose and time of application in relation to antigen, IF can be both immunopotentiating and immunosuppressive in vivo and in vitro (Texas Reports, pp. 357-69). In

In addition, specifically sensitized lymphocytes have been observed to produce IF after contact with antigen. Such antigen-induced IF could therefore be a regulator of the immune response, affecting both circulating antigen levels and expression of cellular immunity (Texas Reports, pp. 370-74). IF is also known to enhance the activity of killer lymphocytes and antibody-dependent cell-mediated cytotoxicity (R.R. Herberman et al., "Augmentation By Interferon Of Human Natural And Antibody-Dependent Cell-Mediated Cytotoxicity", Nature, 277, pp. 221-23 (1979); P. Beverley and D. Knight, "Killing Comes Naturally", Nature, 278, pp. 119-20 (1979); Texas Reports, pp. 375-80; M. Lucero et al., "Induction And Kinetics Of Natural Killer Cells in Humans Following Interferon Therapy", Nature, 282, pp. 417-19 (1979); S. Einhorn et al.,

Acta Med. Scand., 20, pp. 477-83 (1978)). Both may be directly or indirectly involved in the immunological attack on tumor cells.

Therefore, in addition to its use as a human antiviral agent, HIF has potential application in antitumor and anticancer therapy (The Interferon System, pp. 319-21 and 394-99). It is now known that IFs affect the growth of many classes of tumors in many animals (The Interferon System, pp. 292-304). They, like other anti-tumor agents, seem most effective when directed against small tumors. The antitumor effects of animal IF are dependent on dosage and time but have been demonstrated at concentrations below toxic levels. Accordingly, numerous investigations and clinical trials have been and continue to be conducted into the antitumor and anticancer properties of HIFs. These include treatment of several malignant diseases such as osteosarcoma, acute myeloid leukemia, multiple myeloma and Hodgkin's disease (Texas Reports, pp. 429-35). In addition, F IF has recently been shown to cause local tumor regression when injected into subcutaneous tumoral nodules in melanoma and breast carcinoma-affected patients (T. Nemoto et al., "Human Interferons And Intralesional Therapy Of Melanoma And Breast Carcinoma", Amer. Assoc. For Cancer Research, Abs nr. 993, p. 246 (1979)). Significantly, some cell lines which resist the anticellular effects of Le IF remain sensitive to F IF. This differential effect suggests that F IF may be usefully employed against certain classes of resistant tumor cells which appear under selective pressure in patients treated with high doses of

growth of many classes of tumors in many animals (The Interferon System, pp. 292-304). They, like other anti-tumor agents, seem most effective when directed against small tumors. The antitumor effects of animal IF are dependent on dosage and time but have been demonstrated at concentrations below toxic levels. Accordingly, numerous investigations and clinical trials have been and continue to be conducted into the antitumor and anticancer properties of HIFs. These include treatment of several malignant diseases such as osteosarcoma, acute myeloid leukemia, multiple myeloma and Hodgkin's disease (Texas Reports, pp. 429-35). In addition, F IF has recently been shown to cause local tumor regression when injected into subcutaneous tumoral nodules in melanoma and breast carcinoma-affected patients (T. Nemoto et al., Human Interferon And Intralosomal Therapy Of Melanoma And Breast Carcinoma, Amer. Assoc. For Cancer Research, Abs. nr. 993, p. 246 (1979)).

Significantly, some cell lines which resist the anti-cellular effects of Le IF remain sensitive to F IF. This differential effect suggests that F IF may be usefully employed against certain classes of resistant tumor cells which appear under selective pressure in patients treated with high doses of Le IF (T. Kuwata et al., supra; A. A. Creasy et al., Regulatory Functions Of Interferons, N. Y. Acad. Sci., Abstract nr. 17 (1979)). Although the results of these clinical tests are encouraging, the antitumor and anticancer applications of HIF have been severely hampered by lack of an adequate supply of purified HIF.

At the biochemical level IFs induce the formation of at least 3 proteins, a protein kinase (B. Lebleu et al., "Interferon, Double-Stranded RNA And Protein Phosphorylation", Proc. Natl. Acad. Sci. USA, 73, pp. 3107-11 (1976); A. G. Hovanessian and I. M. Kerr, "The (2'-5') Oligoadenylate (ppp A2'-5A2'-5'A) Synthetase And Protein Kinase(s) From Interferon-Treated Cells", Eur. J. Biochem., 93, pp. 515-26 (1979)), a (2'-5')oligo(A) polymerase (A. G. Hovanessian

"The Role of G<sub>0</sub>-G<sub>1</sub> Arrest In The Inhibition Of Tumor Cell Growth By Interferon", Abstract, Conference On Regulatory Functions Of Interferons,

October 23-26,



Several non-bacterial proteins have been obtained in E. coli using recombinant DNA technology.

comprises the steps of producing a single-stranded DNA copy (cDNA) of a purified messenger RNA (mRNA) template for the desired protein; converting the cDNA to double-stranded DNA; linking the DNA to an appropriate site in an appropriate cloning vehicle to form a recombinant DNA molecule and transforming an appropriate host with that recombinant DNA molecule. Such transformation may permit the host to produce the desired protein. These include, for example,

Le IF (C. Weissmann et al., Seminar, Massachusetts Institute of Technology, January 16, 1980). In addition, recombinant DNA technology has been employed to produce a plasmid said to contain a gene sequence coding for F IF (T. Taniguchi et al., "Construction And Identification Of A Bacterial Plasmid Containing The Human Fibroblast Interferon Gene Sequence", Proc. Japan Acad., 55, (Ser. B), pp. 464-69 (1979).

However, in neither of the foregoing has the actual gene sequence of F IF been described and in neither has that sequence been compared to the initial amino acid sequence or amino acid composition of authentic F IF. The <sup>former</sup> Weissmann work is directed only to Le IF, which is distinct chemically, biologically and immunologically from <sup>Class I</sup> F IF (cf. supra). The <sup>later</sup> Taniguchi <sup>report is</sup> results are based solely on hybridization data. These <sup>data</sup> do not enable one to determine if the selected clone contains the complete or <sup>act</sup> gene sequence for F IF or <sup>if</sup> that the <sup>cloned</sup> gene sequence will be able to <sup>express F IF</sup> be expressed in bacteria. Hybridization only establishes that a particular DNA insert is to some extent homologous with and complementary to a mRNA component of the poly(A) RNA that induces interferon activity when injected into pocytes. Moreover, the extent of the homology is dependent on the hybridization conditions chosen for the screening process. Therefore, hybridization to a mRNA component of poly(A) RNA alone does not demonstrate that the selected DNA sequence is a sequence which codes for F IF or a polypeptide which displays the immunological or biological activity of F IF.

### DISCLOSURE OF THE INVENTION

The present invention avoids the uncertainties referred to by providing at least one recombinant DNA molecule characterized by a structural gene whose nucleotide sequence is substantially consistent with the known amino acid composition and sequences of authentic F IF.

By virtue of this invention, it is therefore possible to obtain a structural gene that codes for a polypeptide whose amino acid sequence and composition is substantially consistent with authentic F IF. Replication of these genes in appropriate recombinant DNA molecule-host combinations permits the production of large quantities of these genes. These genes are useful, either as produced in the host or after appropriate derivatization or modification, in compositions and methods for detecting and improving the production of these products themselves *and in selecting other genes related thereto.*

### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic outline of one embodiment of a process of this invention for preparing a mixture of recombinant DNA molecules, some of which are characterized by inserted DNA sequences that characterize this invention.

Figure 2 is a schematic outline of the initial clone screening process of this invention.

Figure 3 is a schematic outline of one embodiment of a clone screening process using DNA sequences prepared in accordance with the invention.

Figure 4 is a restriction map of one of the clones of the invention; the absolute position of each restriction site in this clone has not been determined.

Figure —

DNA Sequence--A linear array of nucleotides connected one to the other by phosphodiester bonds between the 3' and 5' carbons of adjacent pentoses.

Codon--A DNA sequence of three nucleotides (a triplet) which encodes through mRNA an amino acid, a translation start signal or a translation termination signal. For example, the nucleotide triplets TTA, TTG, CTT, CTC, CTA and CTG encode for the amino acid leucine ("Leu"), TAG, TAA and TGA are translation stop signals and ATG is a translation start signal.

Reading Frame--The grouping of codons during translation of mRNA into amino acid sequences. During translation the proper reading frame must be maintained. For example, the sequence GCTGGTTGTAAG may be translated in three reading frames or phases, each of which affords a different amino acid sequence:

GCT GGT TGT AAG--Ala-Gly-Cys-Lys

G CTG GTT GTA AG--Leu-Val-Val

GC TGG TTG TAA G--Trp-Leu-(STOP)

Polypeptide--A linear array of amino acids connected one to the other by peptide bonds between the α-amino and carboxy groups of adjacent amino acids.

Genome--The entire DNA of a cell or a virus. It includes inter alia the structural genes coding for the polypeptides of the substance, as well as operator, promoter and ribosome binding and interaction sequences, including sequences such as the Shine-Dalgarno sequences.

Structural Gene--A DNA sequence which encodes through its template or messenger RNA ("mRNA") a sequence of amino acids characteristic of a specific polypeptide.

Transcription--The process of producing mRNA from a structural gene.

Translation--The process of producing a polypeptide from mRNA.

Expression--The process undergone by a structural gene to produce a polypeptide. It is a combination of transcription and translation.

Plasmid--A nonchromosomal double-stranded DNA sequence comprising an intact "replicon" such that the plasmid is replicated in a host cell. When the plasmid is placed within a unicellular organism, the characteristics of that organism may be changed or transformed as a result of the DNA of the plasmid. For example, a plasmid carrying the gene for tetracycline resistance (Tet<sup>R</sup>) transforms a cell previously sensitive to tetracycline into one which is resistant to it. A cell transformed by a plasmid is called a "transformant".

Phage or Bacteriophage--Bacterial virus many of which consist of DNA sequences encapsidated in a protein envelope or coat ("capsid").

Cloning Vehicle--A plasmid, phage DNA or other DNA sequences which <sup>are</sup> ~~is~~ able to replicate in a host cell, characterized by one or a small number of endonuclease recognition sites at <sup>which</sup> ~~which~~ such DNA sequences may be cut in a determinable fashion without attendant loss of an essential biological function of the DNA, e.g., replication, production of coat proteins or loss of promoter or binding sites, and which contain a marker suitable for use in the identification of transformed cells, e.g., tetracycline resistance or ampicillin resistance. A cloning vehicle is often called a vector.

Cloning--The process of obtaining a population of organisms or DNA sequences derived from one such organism or sequence by asexual reproduction.

Recombinant DNA Molecule or Hybrid DNA--A molecule consisting of segments of DNA from different genomes which have been joined end-to-end outside of living cells and have the capacity to infect some host cell and be maintained therein.

Expression Control Sequence--A sequence of nucleotides that controls and regulates expression of structural genes when operatively linked to those genes.

Referring now to Figure 1, we have shown therein a schematic outline of one embodiment of a process for preparing a mixture of recombinant DNA molecules, some of which include inserted DNA sequences that characterize this invention.

PREPARATION OF POLY(A) RNA CONTAINING HUMAN  
FIBROBLAST INTERFERON mRNA (F IF mRNA)

The RNA used in this invention was extracted from human VGS cells, a diploid fibroblast cell line which can be propagated in monolayer cultures at 37°C. <sup>FIF</sup>Interferon is produced in these cells on induction with poly(I,C) and ~~in the presence of~~ cycloheximide.

For a typical RNA isolation, each of 20 roller bottles of diploid VGS cells in confluent monolayer were "primed" overnight with 100 units/ml F IF and the cultures induced for 1 h with 100 µg/ml poly(I,C) and 50 µg/ml cycloheximide, incubated with cycloheximide (50 µg/ml) for 4 h, harvested by scraping into phosphate-buffered saline and spun down. The cells were lysed for 15 min at 0°C ~~with~~ <sup>by</sup> ~~the use of~~ to remove the intact nuclei containing the DNA and to isolate the cytoplasmic RNA by suspending them in hypotonic buffer (10<sup>-4</sup> M Tris-Cl (pH 7.4), 10 mM NaCl and 1.5 mM MgCl<sub>2</sub>) and adding NP40 to 1%. Nuclei were removed by pelleting in a Sorvall SS-34 rotor for 5 min at 3000 rpm. Sodium dodecyl sulphate <sup>(SDS)</sup> and EDTA were added to the supernatant to 1% and 10 mM, respectively, and the mixture extracted 5 times with 2x vol of 1:1 redistilled phenol and chloroform-isoamyl alcohol (25:1), the aqueous phases containing the RNA being separated by centrifugation in a Sorvall SS-34 rotor at 8000 rpm for 10 min after each extraction. The RNA was precipitated from the aqueous phase by addition of 1/10 vol 2 M sodium acetate (pH 5.1) and 2.5 vol of ethanol. Usually, 60 to 90 µg of total cytoplasmic RNA ~~was~~ <sup>was</sup> obtained per roller bottle.

Other procedures to extract the cytoplasmic RNA have also been used. For example, the cells were totally lysed after homogenization in 0.2 M Tris-<sup>H</sup>Cl (pH 9.0) 50 mM NaCl, 20 mM EDTA and 0.5% <sup>JDS</sup> ~~sodium dodecyl sulphate~~ and extracted with phenol-chloroform as above or the washed cells were suspended in 400  $\mu$ l 0.1 M NaCl, 0.01 M Tris-<sup>H</sup>Cl (pH 7.5), and 0.001 M EDTA ("NTE buffer") and 2.5 ml 4 M guanidinium-isothiocyanate and 1 M  $\beta$ -mercaptoethanol in 20 mM sodium acetate (pH 5.0) were added and the cells homogenized. The lysate was layered on a 1.3-ml 5-7 M CsCl cushion in a Beckman SW-60 Ti nitrocellulose tube, spun for 17 h at 39000 rpm to pellet the RNA and separate it from DNA, proteins and lipids and the RNA extracted once with phenol-chloroform (Reynolds *et al.*, "Interferon Activity Produced By Translation Of Human Interferon Messenger RNA In Cell-Free Ribosomal Systems And In *Xenopus* Oocytes", *Proc. Natl. Acad. Sci. USA*, 72, pp. 4881-4887 (1975) <sup>J.H</sup> ~~Möser~~ *et al.*, "Characterization Of Interferon Messenger RNA From Human Lymphoblastoid Cells", *J. Gen. Virol.*, 44, pp. 231-34 (1979)). <sup>the presence of</sup>


The total RNA was assayed for F IF mRNA by injection into the cytoplasm of *Xenopus laevis* oocytes and determining the <sup>amount of</sup> ~~interferon~~ <sup>F IF</sup> activity induced therein (Reynolds *et al.*, *supra*). The assay was conducted by dissolving the RNA in water and injecting about 50  $\mu$ l into each oocyte. The oocytes were incubated overnight at room temperature in Barth medium (J. Gurdon, "

*J. Embryol. Exper. Morphol.*, 20, pp. 401-14 (1968)), homogenized in part of the medium, the debris removed by centrifugation, and the F IF activity of the supernatant determined. Detection of F IF activity was by reduction of virus-induced cytopathic effect (W. E. Stewart and S. E. Sulkin, "

*Proc. Soc. Exp. Biol. Med.*, 123, pp. 650-53 (1966)). The challenge virus was vesicular stomatitis virus (Indiana

strain) and the cells were human diploid fibroblasts trisomic for chromosome 21 to afford higher F IF sensitivity. F IF activity is expressed relative to the IF reference standard 69/19...

Poly(A) RNA containing F IF mRNA was isolated from the cytoplasmic RNA by adsorption to oligo(dT)-cellulose (type 7; R-L Biochemicals) in 0.4 M NaCl, 10 mM Tris-<sup>H</sup>Cl (pH 7.8), 10 mM EDTA and 0.2% <sup>303</sup> sodium dodecyl sulphate for 10 min at room temperature. RNA aggregation was minimized by heating the RNA for 2 min at 70°C prior to adsorption. After washing the cellulose with the above-mentioned buffer, the poly(A) RNA fraction was eluted with 10 mM Tris-<sup>H</sup>Cl (pH 7.8), 1 mM EDTA and 0.2% <sup>303</sup> sodium dodecyl sulphate. It usually comprised 4-5% of the total RNA, as measured by optical density at 260 nm.

A further purification to enrich the poly(A) RNA in F IF mRNA was effected by formamide-sucrose gradients (T. Pawson *et al.*, "The Size of Rous Sarcoma Virus mRNAs Active in Cell-Free Translation", *Nature*, 268, pp. 416-20 (1977)). These gradients gave much higher resolution than the nondenaturing sucrose gradients. Usually about 80 µg poly(A) RNA was dissolved in 50% formamide, 100 mM LiCl, 5 mM EDTA, 0.2% <sup>303</sup> sodium dodecyl sulphate and 10 mM Tris-<sup>H</sup>Cl (pH 7.4), heated at 37°C for 2 min to prevent aggregation and loaded on a 5-20% sucrose gradient in a Beckman SW-60 Ti polyallomer tube. After centrifugation at 20°C for 4 1/2 h at 60000 rpm in the Beckman SW-60 Ti rotor with total <sup>14</sup>C-labeled eukaryotic RNA serving as size markers, the gradient was fractionated and the optical density of the fractions was determined. All RNA fractions were precipitated twice with 0.5 M NaCl and 2.5 vol ethanol and assayed for interferon mRNA activity as described above.

Alternatively, the oligo(dT)-adsorbed mRNA (60 µg) was fractionated by electrophoresis in a 4% polyacrylamide gel in 7 M urea, 0.1% <sup>303</sup> sodium dodecyl sulphate,

## CLONING OF DOUBLE-STRANDED DNA

A wide variety of host/cloning vehicle combinations may be employed in cloning the double-stranded cDNA prepared in accordance with this invention. For example, useful cloning vehicles may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences, such as various known derivatives of SV40 and known bacterial plasmids, e.g., plasmids from E. coli including col E1, pCR1, pBR322, pMB9 and their derivatives, wider host range plasmids, e.g., RP4, phage DNA, e.g., the numerous derivatives of phage  $\lambda$  e.g., NM 989, and other DNA phages, e.g., M13 and fd, and vectors derived from combinations of plasmids and phage DNAs such as plasmids which have been modified to employ phage DNA or other expression control sequences or yeast plasmids such as the 2  $\mu$  plasmid or derivatives thereof. Useful hosts may include bacterial hosts such as E. coli HB 101, E. coli X1776, E. coli X2282, E. coli MRC1 and strains of Pseudomonas, Bacillus subtilis, Bacillus stearothermophilus and other bacilli, yeasts and other fungi, animal or plant hosts such as animal (including human) or plant cells in culture or other hosts. Of course, not all host/vector combinations may be equally efficient. The particular selection of host/cloning vehicle combination may be made by those of skill in the art after due consideration of the principles set forth without departing from the scope of this invention.

Furthermore, within each specific cloning vehicle, various sites may be selected for insertion of the double-stranded DNA. These sites are usually designated by the restriction endonuclease which cuts them. For example, in pBR322 the PstI site is located in the gene for  $\beta$ -lactamase, between the nucleotide triplets that code for amino acids 181 and 182 of that protein. This site was employed by C. Weissmann et al., supra, in their synthesis of polypeptides displaying an immunological or biological activity of L<sup>1</sup>IF. One of the two HindII endonuclease recognition sites is between the triplets coding for amino acids 101 and 102 and one of the several Taq sites at the triplet coding for amino acid 45 of  $\beta$ -lactamase in pBR322. In similar fashion, the EcoRI site and the PvuII site in this plasmid lie outside of any coding region, the EcoRI site being located between the genes coding for resistance to tetracycline and ampicillin, respectively. This site was employed by T. Taniguchi et al., supra, in



their recombinant synthetic scheme. These sites are well recognized by those of skill in the art. It is, of course, to be understood that a cloning vehicle useful in this invention need not have a restriction endonuclease site for insertion of the chosen DNA fragment. Instead, the vehicle could be joined to the fragment by alternative means.

The vector or cloning vehicle and in particular the site chosen therein for attachment of a selected DNA fragment to form a recombinant DNA molecule is determined by a variety of factors, e.g., number of sites susceptible to a particular restriction enzyme, size of the protein to be expressed, susceptibility of the desired protein to proteolytic degradation by host cell enzymes, contamination of the protein to be expressed by host cell proteins difficult to remove during purification, expression characteristics, such as the location of start and stop codons relative to the vector sequences, and other factors recognized by those of skill in the art. The choice of a vector and an insertion site for a particular gene is determined by a balance of these factors, not all selections being equally effective for a given case.

Although several methods are known in the art for inserting foreign DNA into a cloning vehicle or vector to form a recombinant DNA molecule, the method preferred in accordance with this invention is characterized by digesting the plasmid (in particular pBR322) with that restriction enzyme specific to the site chosen for the insertion (in particular PstI) and adding dA tails to the 3' termini by terminal transferase. In similar fashion, the double-stranded cDNA is elongated by the addition of dT tails to the 5' termini to allow joining to the tailed plasmid. The tailed plasmid and cDNA are then annealed to insert the cDNA in the appropriate site of the plasmid and to circularize the hybrid DNA, the complementary character of the tails permitting their cohesion (Figure 1). The resulting recombinant DNA molecule now carries a gene at the chosen <sup>site</sup> restriction site (Figure 1). This method of dA-dT tailing for insertion is described by D.A. Jackson et al., "Biochemical Methods for Inserting New Genetic Information Into DNA of Simian Virus 40: Circular SV40 DNA Molecules Containing Lambda Phage Genes And The Galactose Operon of Escherichia coli", Proc. Natl. Acad. Sci. USA, 69, pp. 2904-2909 (1972) and R. Devos et al., supra. It results in about 3

*check  
has been?*

prepared. Again, only a very few of these clones will contain the gene for FIP or fragments thereof (Figure 1). The preferred host in accordance with this invention is E. coli BH 101.

1. Preparation of PstI-Cleaved, dGMP-elongated pBR322

Plasmid pBR322 (23<sup>4</sup>) was digested ~~with 23 units~~ <sup>completely with</sup> PstI endonuclease (New England Biolabs) in ~~100~~ <sup>10</sup> mM Tris-HCl (pH 7.6), <sup>7</sup> mM MgCl<sub>2</sub>, ~~50 mM NaCl~~, <sup>7</sup> mM 2-mercaptoethanol, ~~300 mg/ml~~ <sup>10</sup> bovine serum albumin ("BSA") (Calbiochem). After ~~3 h at 37°C~~, the mixture was extracted ~~several times~~ with 1 vol phenol and ~~1-5~~ <sup>10</sup> vol ether and precipitated with 2.5 vol ethanol; ~~0.2 M sodium acetate solution~~.

Addition of homopolymeric dA tails (Figure 1) by terminal deoxynucleotidyl transferase (TdT) (purified according to L. Chang and F.J. Bollum, "Deoxynucleotide-Polymerizing Enzymes Of Calf Thymus Gland", J. Biol. Chem., 246, pp. 909-16 (1971)) was done in a 50- $\mu$ l reaction volume containing 0.14 M potassium cacodylate, 30 mM Tris-HCl (pH 6.8), 1 mM  $\text{CaSO}_4$ , 0.2  $\mu$ g/ $\mu$ l heat-inactivated bovine serum albumin, 0.8 mM DTT, 0.2 mM dATP and some  $\alpha$ -<sup>32</sup>P-dATP. Incubation was at 37°C for 5 min before EDTA was added to 10 mM and SDS to 0.1 % and the mixture extracted with phenol and chromatographed on Sephadex G50 in TE buffer. The void fractions, containing the linearized and elongated pBR322, were further purified by adsorption in 10 mM Tris-HCl (pH 7.8), 1 mM EDTA and 0.4 M NaCl to oligo(dT)cellulose. After extensive washing, the desired fractions were eluted with 10 mM Tris-HCl (pH 7.8) and 1 mM EDTA.

2. Preparation of dT-elongated DNA

Double-stranded DNA was elongated with dTMP residues in similar fashion to that described above for dA tailing of pBR322, except that dTTP and some <sup>3</sup>H-dTTP replaced the dATP and  $\alpha$ -<sup>32</sup>P-ATP. Purification on oligo(dT)cellulose was, of course, omitted. As before, the dT-elongated DNA is a mixture of different species, only a very few of which are IF-related (Figure 1).

3. Preparation of Ca<sup>++</sup>-Treated E.coli HB101

Ca<sup>++</sup>-treated E.coli HB101 was prepared by the method of

① E.M. Lederberg and S.N. Cohen, "Transformation of Salmonella Typhimurium by Plasmid Deoxyribonucleic Acid",

② J. Bacteriol., 119, pp. 1072-74 (1974) by inoculating the E.coli HB101 (a gift from H. Boyer) into 5 ml LB medium (10 parts bacterio-tryptone, 5 parts yeast extract and 5 parts NaCl per liter) and cultures grown overnight at 37°C. The fresh cultures were diluted 1/100 in 20 ml LB medium and grown to a density of about  $2 \times 10^8$  bacteria per ml, quickly chilled in ice and pelleted at 6000 rpm for 5 min in a Sorvall SS34 rotor at 4°C. The cells, kept at 0-4°C, were washed with 20 ml 100 mM MgCl<sub>2</sub>, repelleted by centrifugation and suspended in 10 ml 100 mM CaCl<sub>2</sub>. After 20 min in ice, the cells were repelleted and resuspended in 2 ml 100 mM CaCl<sub>2</sub> and maintained at 0°C for 15 min. Aliquots (200 µl), supplemented with glycerol to 11%, could be stored for several months at -80°C without loss of activity (D.A. Morrison, "Transformation in Escherichia coli : Cryogenic Preservation of Competent Cells", J. Bacteriol., 132, pp. 349-51 (1977)).

#### 4. Annealing of dA-elongated pBR322 and dT-elongated DNA

The vector's and DNA insert's complementary dA- and dT-tails permit annealing to form the desired hybrid plasmid or recombinant DNA molecule. For this purpose, the dA-tailed PstI-cleaved pBR322 vector and the mixture of sized dT-tailed cDNAs were dissolved in TSE buffer (10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 100 mM NaCl) to 1.5 µg/ml plasmid and to a molar ratio of plasmid to DNA insert of 1.5 to 2.0. After heating to 65°C for 10 min, the mixture was cooled slowly to room temperature over 4 h.

The product is, of course, a large mixture of different recombinant DNA molecules and some cloning vehicles without inserted DNA sequences. However, each recombinant DNA molecule contains a cDNA segment at the PstI site. Each such cDNA segment may comprise a gene or a fragment thereof. Only a very few of the cDNA segments code for FIF or a portion thereof (Figure 1). The vast majority code for one of the other proteins or portions thereof whose mRNA's were part of the poly(A) RNA used in the process of this invention (Figure 1).

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5. Transfection Of *E. coli* ~~HB101~~ With The Annealed Hybrid Plas-  
mids

P3 containment facilities were used for the transfection process and all subsequent steps in which the resulting transformed bacteria were handled. Aliquots (90  $\mu$ l or less) of the above mixture were cooled to 0°C and 1 M  $\text{CaCl}_2$  added to 0.1 M. Aliquots (100  $\mu$ l or less) of this solution were added to 200  $\mu$ l  $\text{Ca}^{++}$ -treated *E. coli* HB101 in ice and after standing at 0°C for 30 min, the cells were heat-shocked for 5 min at 37°C and cooled again at 0°C for 15 min. After addition of 2 ml LB-medium, the cells were incubated at 37°C in a shaking water bath for 30 to 45 min and the bacterial suspension plated out onto 1.2% agar plates, containing LB medium supplemented with 10  $\mu$ g/ml tetracycline.

Since plasmid pBR322 includes the gene for tetracycline resistance, *E. coli* hosts which have been transformed with a plasmid having that gene intact will grow in cultures containing that antibiotic to the exclusion of those bacteria not so transformed. Therefore, growth in tetracycline-containing culture permits selection of hosts transformed with a recombinant DNA molecule or recyclized vector.

After 24 h at 37°C, individual colonies were picked and suspended in 100  $\mu$ l LB medium (supplemented as above) in the wells of microtiter plates (Dynatech). After incubation at 37°C overnight, 11  $\mu$ l dimethylsulfoxide were mixed into each well and the trays sealed with adhesive tape. The plates were stored at -20°C and a library of 17,000 individual clones of transformed *E. coli* HB101 was prepared. This library was derived from 270 fmoles (128 ng) dT-tailed cDNA inserts, which in turn were synthesized from 4.4  $\mu$ g gradient purified poly(A) RNA. About 98% of the clones of this library (band on representative fractions) were sensitive to carbanicillin (a more stable ampicillin-derivative). Therefore, about 98% of the library contained a plasmid having an insert in the PstI-site of the  $\beta$ -lactamase gene of pBR322 only about 2% contained a recyclized vector without insert.

These 17,000 clones contain a variety of recombinant DNA molecules representing complete or partial copies of the mixture of mRNAs in the poly(A) RNA preparation from FIF-producing cells (Figure 2). The majority of these will contain only a single recombinant DNA molecule. Only a very few of these recombinant

DNA molecules are related to FIF. Accordingly, the clones must be screened to separate the FIF-related clones from the others.

## SCREENING FOR A CLONE CONTAINING F IFcDNA

There are several approaches to screen for bacterial clones containing <sup>F IF</sup> interferon cDNA. ~~These include, for example, RNA selection hybridization (Alwine et al., infra), differential hybridization (T.P. St. John and R.W. Davis, "Isolation of Galactose-Inducible DNA Sequences From Saccharomyces Cerevisiae By Differential Plaque Filter Hybridization", Cell, 16, pp. 443-452 (1979));~~ ~~Hybridization with a synthetic probe (B. Noyes et al., "Detection And Partial Sequence Analysis Of Gastrin mRNA By Using An Oligodeoxynucleotide Probe", Proc. Natl. Acad. Sci. USA, 76, pp. 1770-1774 (1979))~~ or screening for clones that produce the desired protein by immunological (A.C.Y. Chang et al., <sup>assays</sup>) assays. We have chosen RNA selection hybridization as being the most convenient and promising method for primary screening.

### A. RNA Selection Hybridization Assay

#### 1. Overview Of The Initial Assay

Referring now to Figure 2, <sup>the</sup> recombinant DNA <sup>molecules were</sup> was isolated from a culture of a mixture of about 46 clones sensitive to carbenicillin and resistant to tetracycline from the above library of clones (two mixtures of 2 clones shown in Figure 2) (Step A). The recombinant DNA molecules were cleaved, ~~denatured~~ and hybridized to total RNA containing F IFmRNA prepared as before (Step B). All recombinant DNA molecule-total RNA hybrids were separated from the non-hybridized total RNA (Step C). The <sup>hybridized</sup> total RNA was recovered from the hybrids and purified (Step D). The recovered RNA was assayed for F IFmRNA activity as above (Step E). If, and only if, the mixture of recombinant DNA molecules contains a recombinant DNA molecule having an inserted nucleotide sequence capable of hybridizing to the F IFmRNA in the total RNA, under stringent hybridization conditions, will the mRNA released from that hybrid cause the formation of F IF in oocytes, because mRNA released from any other recombinant DNA molecule-total RNA hybrid will not be F IF-related. If a group of 46 clones gave a positive response, the clones were regrouped <sup>subgroups</sup> in 4 lots of 8 and 2 lots of 7, and

into 6 subgroups ( )

<sup>subgroup</sup>  
each ~~lot~~ assayed as before. This process was continued until a single clone responding to this assay was identified.

There is no assurance that the recombinant DNA molecules and bacterial <sup>cultures</sup> ~~clones~~ transformed therewith, which are thus identified, contain the complete F IFcDNA sequence of F IF or even that the DNA sequence actually codes for F IF. However, the recombinant DNA molecules will certainly contain extensive nucleotide sequences complementary to the F IFmRNA coding sequence. Therefore, the recombinant DNA molecule may at least be used as a source of a probe to screen rapidly other recombinant DNA molecules and clones transformed with them to identify further sets of clones which may contain <sup>nucleotide</sup> an authentic and complete F IF nucleotide coding sequence. ~~These sequences of these by inserted DNA fragment of these hybrid plasmids may also be determined and compared to~~ <sup>the amino acid composition and initial sequence reported for authentic F IF (1980)</sup>

## 2. Execution Of The Initial Assay

### Step A - Preparation Of The Recombinant DNA Molecule Mixture

Replicas of a microtiter plate containing 96 clones from the above library of clones were made on LB-agar plates, one containing 10 µg/ml tetracycline and the other supplemented with 100 µg/ml carbenicillin. In this manner, two sets of about 45 <sup>46</sup> clones, resistant to tetracycline and sensitive to carbenicillin, were picked and grown <sup>separately</sup> overnight at 37°C in 100 ml LB medium, containing 10 µg/ml tetracycline. These cultures were pooled, spun down in a Sorvall GS-3 rotor at 8000 rpm for 10 min, washed twice with TES buffer (50 mM Tris-HCl (pH 8), 5 mM EDTA, 5 mM NaCl) and resuspended in 40 ml TES per 1 of initial culture volume. The cells were lysed with lysozyme-Triton X-100 (M. Kahn et al., "Plasmid Cloning Vehicles Derived From Plasmids ColEI, F, R6K And RK2", in Methods of Enzymology, 68, : Recombinant DNA (R. Wu, ed.) (1980) in press). Forty ml of the TES suspended cells were combined <sup>with</sup> 20 ml 10% sucrose in 50 mM Tris-HCl (pH 8) and lysozyme to 1.3 mg/ml and allowed to stand at room temperature for 20 min. To this suspension were added 1 ml 0.5 M EDTA-NaOH (pH 8), 8 ml 0.2% Triton X-100, 25 mM EDTA, 50 mM Tris-HCl (pH 8) and the lysis completed at room temperature for 30 min. Cellular debris and most of the chromosomal DNA were removed by pelleting in a Beckmann SW27 rotor at 24000 rpm for 45 min. The supernatant was

cooled in ice, combined with 1/3 vol 40% polyethylene glycol 6000 - 2 M NaCl and allowed to stand overnight at 0°C. The resulting precipitate was collected in a Sorvall HB4 rotor at 5000 rpm for 10 min at 4°C and dissolved in TES buffer. The solution, with 0.2 vol 10 mg/ml ethidium bromide (Serva) and CsCl to 1 g/ml, was centrifuged in a Beckmann R60 Ti-rotor at 40000 rpm for at least 48 h, one polyallomer tube usually being sufficient for the lysate from 1-2 l of original culture volume. Two DNA bands could be visualized in the tube <sup>under</sup> by UV-illumination. The band of highest density corresponds to plasmid form I DNA, the second band corresponds to form II and form III plasmid DNAs and some chromosomal DNA. The first band was cut from the tube, ethidium bromide removed by six isoamyl alcohol extractions, and the aqueous phase diluted with 3 vol water-supplemented with up to 0.2 M sodium acetate (pH 5.1) before DNA precipitation with 2.5 vol ethanol. The DNA was redissolved, extracted with phenol and again precipitated with ethanol. The quality of the DNA was monitored by electrophoresis on a 1% agarose gel in 40 mM Tris-HCl (pH 7.8), 20 mM sodium acetate, (ethidium bromide staining). If the DNA ~~contained~~ was contaminated with form II or form III DNAs, it was further purified by neutral sucrose-gradient centrifugation: 300 µg DNA in 10 mM Tris-HCl (pH 7.6) and 1 mM EDTA was loaded on a 36-ml 5-20% sucrose gradient in 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1 M NaCl, centrifuged in polyallomer tubes for 16 h at 24000 rpm in a Beckmann SW27-rotor at 18°C and the DNA containing fractions (OD<sub>260</sub>) pooled and precipitated with sodium acetate-ethanol.

2 mg/cm<sup>2</sup>, followed by

#### Step B - Hybridization Of The DNA With Total RNA

About 150 µg DNA, thus prepared, was combined with some uniformly labelled <sup>32</sup>P-marker DNA and 2 µg pSTNV-1 DNA (a recombinant plasmid containing a full size cDNA copy of satellite tobacco necrosis virus ("STNV")-RNA; J. Van Emmelo et al., "Construction And Characterization Of A Plasmid Containing A Nearly Full-Size DNA Copy Of Satellite Tobacco Necrosis Virus RNA", J. Mol. Biol., submitted for publication) as internal control, sheared by sonication in an MSE sonicator and precipitated with sodium acetate-ethanol.

A diazobenzylloxymethyl (DBM)-cellulose solid matrix (cf., J.C. Alwine et al., "Method For Detection Of Specific RNAs In Agarose Gels By Transfer To Diazobenzyl Oxymethyl Paper and Hybridization With DNA Probes", Proc. Natl. Acad. Sci. USA, 74,



pp. 5350-54 (1977)) was prepared according to the method of J.C. Alwine et al., "Detection of Specific RNAs by Specific Fragments of DNA," Method in Enzymology, 68: Recombinant DNA (R. Wu, ed.) (1980) (in press). For a paper matrix, a sheet of Whatman 540 paper was evenly soaked in 2-3 ml 1-(m-nitrobenzyloxy)methyl pyridinium chloride (NEPC/BDH) - 0.7 ml sodium acetate trihydrate - 2.8  $\mu$ l water per  $\text{cm}^2$ , incubated at 60°C until dry and for a further 10 min, and baked at 130-135°C for 30-40 min. After washing several times with water (about 20 min), 3 times with acetone (about 20 min) and drying, the paper was incubated at 60°C for 30 min in 0.4 ml 20% sodium dithionite-water with occasional shaking. The paper was again washed four times with water, once with 30% acetic acid for 5 min and four times with water, transferred for 30 min at 0°C to 0.3 ml per  $\text{cm}^2$  ice-cold 1.2 M HCl to which 10 mg/ml fresh  $\text{NaNO}_2$  had been added immediately before use, and washed twice quickly with ice-cold water and once with 80% dimethyl sulfoxide (spectrophotometric grade, Merck) - 20% 25 mM sodium phosphate (pH 6.0). For a powder matrix essentially the same procedure was followed using micro granular cellulose powder (Whatman CC31), the quantities being expressed against the corresponding weight of the cellulose matrix.

Initially, we used a powder matrix because the capacity for binding was higher, so relatively smaller volumes for hybridization, washes and elution could be used. Subsequently, we used a paper matrix for individual clone screening. Use of paper permits efficient elution with water which proved superior for the later assay of F IPmRNA. (for powder) (for paper)

The DNA (50-100  $\mu$ g for powder 3-4  $\mu$ g for paper) prepared above was dissolved in 25 mM sodium phosphate (pH 6.0) heated for 1 min, chilled and four vol DMSO added. Coupling to the matrix (50 mg or a disc (20 mm dia.)) usually proceeded over a weekend at 4°C with continuous mixing. The volume of the DNA was kept rather small to allow close contact with the matrix and thereby enhance efficient coupling of the DNA to the matrix. After coupling, the matrix was washed four times with water, four times with 0.4 N NaOH at 37°C for 10 min each, again four times with water at room temperature and finally twice with hybridization buffer (50% formamide (deionized, Baker), 40 mM piperazine-N,N'-bis(2-ethane sulfonic acid) (pH 6.4) ("PIPES", Sigma), 1 mM EDTA, 0.6 M NaCl and 0.1% SDS) at 4°C. Coupling efficiencies were measured by  $^{32}\text{P}$ -radioactivity.

Twenty  $\mu$ g total RNA, prepared as before, and 50 ng STNV-RNA were dissolved in 250  $\mu$ l (50  $\mu$ l for paper matrix) hybridization buffer and added to the DNA coupled matrix. The matrix was heated to 70°C for 2 min and held at 37°C overnight with gentle mixing.

#### Step C - Separation Of Hybridized Total RNA-DNA From Non-Hybridized Total RNA

After centrifugation of ~~the~~ <sup>g</sup>above powder matrix, the unhybridized RNAs were removed and the matrix washed seven times with (0.28 ml) 50% formamide, 10 mM PIPES (pH 6.4), 1 mM EDTA, 0.3 M NaCl and 0.1% SDS, the lower salt content of these washes destabilizing non-specific RNA-DNA binding. Each wash was followed by centrifugation and resuspension of the matrix in the buffer. For subsequent assay, the first wash was pooled with the unhybridized DNA ("Fraction 1") and washes 2-4 ("Fraction 2") ~~as~~ <sup>and</sup> washes 5-7 ("Fraction 3") were pooled. In these hybridization<sup>s</sup> to a paper matrix, a similar procedure was ~~employed~~ <sup>utilized</sup> except that <sup>0.1% salt</sup> water ~~was~~ used for each wash.

#### Step D - Purification Of Hybridized Total RNA

*Following that*  
The hybridized total RNA-DNA was eluted from <sup>a</sup>the powder matrix with 900  $\mu$ l 99% formalde<sup>a</sup>hyde, 0.2% SDS at 70°C for 2 min and chilled in ice (A.G. Smith, personal communication). The hybridized total RNA-DNA was eluted from <sup>a</sup>the paper matrix by <sup>first washing with</sup> 100  $\mu$ l of ice cold water and two 150  $\mu$ l water elutions at 80°C for 2 min. For subsequent assay these elutions and the 100  $\mu$ l wash were pooled ("Fraction 4").

To one-half of each fraction, 0.1  $\mu$ g calf liver tRNA or ribosomal RNA were added (Fractions 1A, 2A, 3A and 4A) and to the other half 8  $\mu$ g eukaryotic poly(A) RNA or ribosomal RNA were added (Fractions 1B, 2B, 3B, 4B). The fractions were <sup>precipitated by</sup> ~~precipitated~~ by the addition of 0.5 M NaCl and 2.5 vol ethanol to remove traces of formamide and other impurities.

#### Step E - Determination Of F IFmRNA Activity

Fractions 1A, 2A, 3A and 4A were translated in <sup>25  $\mu$ l</sup> nuclease-

After incubation, 25

(A)

Twenty-five  $\mu$ l of cell lysate, from above, were combined

with 1  $\mu$ l 10% dioxycolate - 10% Triton X100 and 2  $\mu$ l anti serum - PBS

(1:9) and was heated at 37°C for 1h. Twenty  $\mu$ l Staphylococcus

Rapid Isolation OF

aureus Cowan I (freshly worked, S.W. Kessler et al., "Antigens From Cells WITH A Staphylococcal Protein A-Antibody Adsorbent: Parameters

) in 10% 100mM NaCl, 10mM Tris-HCl (pH 7.4), 1mM

EDTA, 0.05% NP40 was added and the mixture maintained at 20°C

for 30min and centrifuged in an Eppendorf 5412 centr. tube for 2 min.

The pellet was washed and centrifuged twice with PBS and the

Final pellet dissolved in sample buffer and electrophoresed in 13%

polyacrylamide gels described by U.K. Laemmli et al., "Cleavage of Structural Proteins During The Assembly OF THE Head OF Bacteriophage T4"

Using Interaction of Antibody-Antigen complexes with Protein A"

PP-617-1634 K19351

J. Immunology, 115

32  
T-27A  
(prepared according to the procedure)  
treated rabbit reticulocyte lysate (R.B. Pelham and R.J. Jackson, "An Efficient mRNA-Dependent Translation System For Reticulocyte Lysates", Eur. J. Biochem., 7, pp. 247-56 (1976)) in the presence of <sup>35</sup>S-methionine, immunoprecipitated with antiserum, and electrophoresed in 13% polyacrylamide gel (V.K.R. 1977).

Nature, 227, pp. 680-685 (1970), and autoradiographed. Comparison of the STNV-RNA translation products in Fractions 1A and 4A provide an indication of the efficiency of hybridization and RNA degradation in the process. Fractions 1B, 2B, 3B and 4B were dissolved in 2 µl water and assayed in oocytes for F IFmRNA content as described above.

### 3. Subsequent Assay - Hybridization To Nitrocellulose Sheets

Some subsequent assays of individual clones were done on nitrocellulose sheets (M. Crockett et al., "Cloning Of An Almost Full-Length Chicken Conalbumin Double-Stranded cDNA",

Nucleic Acids Res., 6, pp. 2435-2452 (1979)). The DNA was dissolved in 2M NaCl and 0.2 M NaOH, heated at 100°C for 1 min, chilled, and spotted on detergent free Millipore filter (pore size 0.45 µm; 1 mm dia.). The filters were baked for 2 h at 80°C, washed in 0.3 M NaCl, 2 mM EDTA, 0.1% SDS, 10 mM Tris-HCl (pH 7.5) and dried at room temperature. The RNA was hybridized for 3 h at 47°C in 30% formamide, 0.5 M NaCl, 0.4% SDS, 2 mM EDTA, 50 mM PIPES (pH 7.5). Hybridization was stopped by dilution with 10 vol 0.1 M NaCl and the filters were washed several times in 15 ml of 0.3 M NaCl, 0.1% SDS, 2 mM EDTA, 10 mM Tris-HCl (pH 7.5) by shaking at 45°C and several times in the same solution without SDS at 4°C. Elution of the hybridized RNA-DNA was effected in 30 µl 5 mM potassium chloride at 100°C for 1 min.

### 4. Results Of The RNA Selection Hybridization Assay

Sixteen groups of about 46 clones were screened (Groups A-P). In six of the groups, Fraction 1B contained the only F IFmRNA activity, in eight of the groups no F IFmRNA was detected and in two groups (Groups C and P) F IFmRNA was observed in Fraction 4B. The positive assays are reported in the following format: log-arithm of F IF units (calibrated against reference standard 69/19),

(2/6) 549-599 66 28, 82, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 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883, 884, 885, 886, 887, 888, 889, 890, 891, 892, 893, 894, 895, 896, 897, 898, 899, 900, 901, 902, 903, 904, 905, 906, 907, 908, 909, 910, 911, 912, 913, 914, 915, 916, 917, 918, 919, 920, 921, 922, 923, 924, 925, 926, 927, 928, 929, 930, 931, 932, 933, 934, 935, 936, 937, 938, 939, 940, 941, 942, 943, 944, 945, 946, 947, 948, 949, 950, 951, 952, 953, 954, 955, 956, 957, 958, 959, 960, 961, 962, 963, 964, 965, 966, 967, 968, 969, 970, 971, 972, 973, 974, 975, 976, 977, 978, 979, 980, 981, 982, 983, 984, 985, 986, 987, 988, 989, 990, 991, 992, 993, 994, 995, 996, 997, 998, 999, 1000, 1001, 1002, 1003, 1004, 1005, 1006, 1007, 1008, 1009, 1010, 1011, 1012, 1013, 1014, 1015, 1016, 1017, 1018, 1019, 1020, 1021, 1022, 1023, 1024, 1025, 1026, 1027, 1028, 1029, 1030, 1031, 1032, 1033, 1034, 1035, 1036, 1037, 1038, 1039, 1040, 1041, 1042, 1043, 1044, 1045, 1046, 1047, 1048, 1049, 1050, 1051, 1052, 1053, 1054, 1055, 1056, 1057, 1058, 1059, 1060, 1061, 1062, 1063, 1064, 1065, 1066, 1067, 1068, 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assay of Fraction 1B (background) <sup>and</sup> assay of Fraction 4B.

| <u>Group</u> | <u>Fraction 1B</u> | <u>Fraction 4B</u> |
|--------------|--------------------|--------------------|
| C            | 1.0                | 0                  |
|              | 0.5                | 0.5                |
|              | 0                  | 0.2                |
| O            | 0                  | 0                  |
|              | 0.2                | 0.5                |

(Subgroups)  
Group O was subdivided into 6 subgroups <sup>e</sup> O<sub>1</sub> to O<sub>6</sub>; (four of eight clones and two of seven clones) and hybridized and assayed as before, except a 400 ml culture per clone was used. The subgroups gave the following results, presented in the same format as above:

| <u>Subgroup</u> | <u>Fraction 1B</u> | <u>Fraction 4B</u> |
|-----------------|--------------------|--------------------|
| O <sub>1</sub>  | 0                  | 1.2                |
|                 | 0                  | 1.5                |
|                 | 0                  | 0.5                |
|                 | 0                  | 0.5                |
|                 | 0.2                | 0.5                |
|                 | 0                  | 1.2*               |
| O <sub>2</sub>  | 0.1                | 0                  |
| O <sub>3</sub>  | 0.7                | 0                  |
|                 | 0.5                | 0                  |
| O <sub>4</sub>  | 0                  | 0                  |
| O <sub>5</sub>  | 0.5                | 0                  |
| O <sub>6</sub>  | 0                  | 0                  |

\* DPM paper method

<sup>0.1/1</sup> Subgroup O<sub>1</sub> was subdivided into its individual clones (designated clones <sup>0.1/1</sup> 1-4) and hybridized and assayed as before, except a 700 ml culture per clone was used:

| <u>Clone</u>     | <u>Fraction 1B</u> | <u>Fraction 4B</u> |
|------------------|--------------------|--------------------|
| O <sub>1/1</sub> | 0.2                | 0                  |
|                  | 0.7                | 0                  |
|                  | 0.7                | 0*                 |
|                  | 1.0                | 0**                |

|           | <u>Fraction 1B</u> | <u>Fraction 4B</u>  |
|-----------|--------------------|---------------------|
| $O_{1/2}$ | 1.2                | 0                   |
|           | 0.2                | 0 <sup>W</sup>      |
|           | 0.7                | 0 <sup>W</sup>      |
| $O_{1/3}$ | 1.2                | 0                   |
|           | 1.0                | 0.2 <sup>W</sup>    |
|           | 1.2                | 1.0(?) <sup>W</sup> |
|           | 1.2                | 0 <sup>W</sup>      |
| $O_{1/4}$ | 1.2                | 0                   |
|           | 1.2                | 0                   |
|           | 1.0                | 0 <sup>W</sup>      |
|           | 1.2                | 0 <sup>W</sup>      |
| $O_{1/5}$ | 0.7                | 0                   |
|           | 0.7                | ≤ 0.2 <sup>W</sup>  |
|           | 1.0                | 0 <sup>W</sup>      |
| $O_{1/6}$ | 0.7                | 0                   |
|           | 1.0                | ≤ 0.2 <sup>W</sup>  |
|           | 0.5                | 0 <sup>W</sup>      |
| $O_{1/7}$ | 0.5                | 0                   |
|           | 1.2                | 0 <sup>W</sup>      |
|           | < 0.2              | 0.5 <sup>W</sup>    |
| $O_{1/8}$ | 0                  | 1.7 <sup>W</sup>    |
|           | < 0.2              | 1.2 <sup>W</sup>    |
|           | 0                  | 0.7 <sup>W</sup>    |
|           | 0                  | 1.0 <sup>W</sup>    |

W DPM paper method

W<sup>W</sup> Nitrocellulose sheets

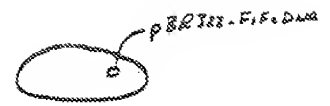
Therefore, clone  $O_{1/8}$  contains a recombinant DNA molecule capable of hybridizing F IFmRNA from total RNA containing F IFmRNA. Non-specific RNA-DNA binding is unlikely, because a comparison of Fractions 1A and 4A revealed substantially no non-specific binding of STLV DNA.

Fig. 3



X-Ray  
Exposure

Negative Response



32P-p HFI-F1 - Hinf 2  
Fragment



32P-p HFI-F1 - Hinf  
Fragment

X-Ray  
Exposure

Positive Response

Rik → 1 digit for sequence

This is EXHIBIT FIERS-11  
to  
the Affidavit of Walter C. Fiers  
sworn before me  
this 19th day of November, 2001

Jan → 1 class -

Commissioner for Oath or Notary Public

Erik = - cl3 Hya Sun 3A Huf Shu

→ Hya II →

{ - tail  
- Bal + tail

→  
AVG

sent FIF 20  
AB

- 4 3' end 240 med  
190 mg 570 med  
Σ 810 med

cl3  
7

I 3-4

SRKB

SRKB + FIF

Bth + FIF

800 med →

Dec 30

02

Green

M. L. L. L.

Alto says met Penelline



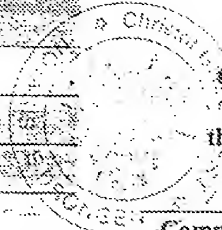
1. Sequence

Rsa

Hpa I

Hpa II

order?



This is EXHIBIT FIERS-12

to

the Affidavit of Walter C. Fiers  
sworn before me

this 19th day of November, 2001

Commissioner for Oath or Notary Public

2. Restrictions

cl 3

+

order?

Hpa int?

EcoR?

orientate

Pan I - Pan II

Hpa I - Hpa II

(cells under need get out)

of Hpa I - Rg

mutual inter clones in screening

3. Expression

(A)

cl 3

before incorporation

Latitudes

(B)

in Hpa II in SRK

mutant - suitable protein

orientate

|                                                         |        |       |
|---------------------------------------------------------|--------|-------|
| Opened                                                  | Dec 30 | 20 02 |
| Décauchetée le                                          |        |       |
|                                                         |        |       |
| Commissioner of Patents<br>Commissaire des brevets      |        |       |
| In presence of examiner<br>en présence de l'examinateur |        |       |

I-IF

detritie

I-IF on MPA

beading

RNA at trans

stimulate

trans + black buffy coat

screening

black lymphocytes

100 - 500

to be prepared

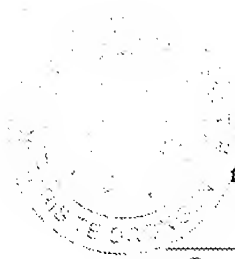
clones

STVV  
F-IF

Dink

Rob. Dwyer  
Laboratory  
Voluntary Biology  
2/10/01

Dec 30 02  
[Signature]  
In presence of [Signature]  
en présence de l'exhibiteur



This is EXHIBIT FIERS-13  
to  
the Affidavit of Walter C. Fiers  
sworn before me  
this 9<sup>th</sup> day of November, 2001

Commissioner for Oath or Notary Public

[Signature]

SEP 12B 2. ... but PHFIP-1 is not fragment, ...  
 about com. ...

by condensation of ... (see sep 12C  
 of ...)

2 filters ... 3 pp 2000

2 DNA PHFIP-2  
 2 DNA PHFIP-2

by condensation to ...

... of ...

| PHFIP-2 | ET | W <sub>2</sub> | W <sub>L</sub> | ET  |
|---------|----|----------------|----------------|-----|
|         | 0  | 0.2            | 0.2            | 2.2 |

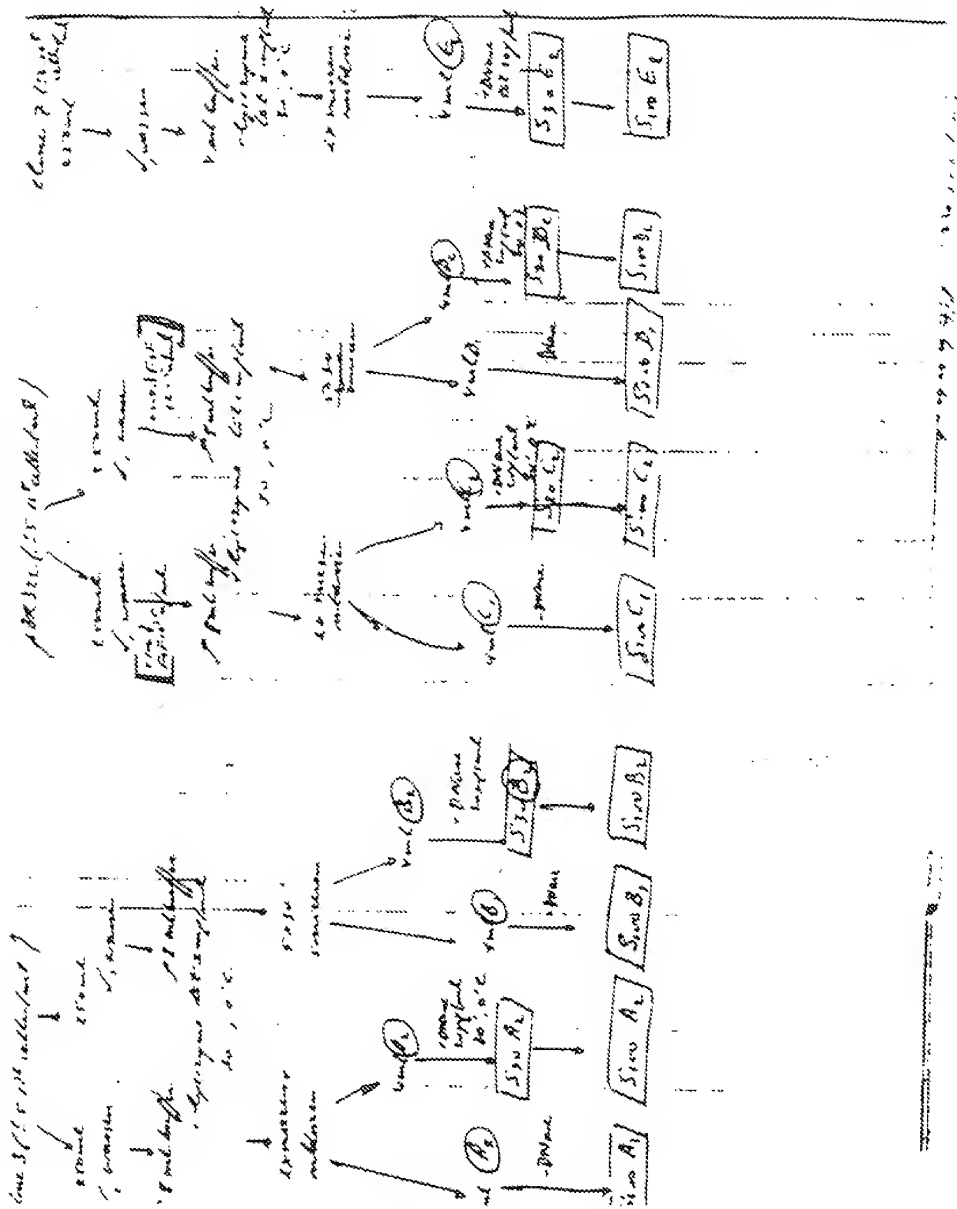
|         |   |   |   |     |
|---------|---|---|---|-----|
| PHFIP-3 | 0 | 0 | 0 | 2.5 |
|---------|---|---|---|-----|

... different ...  
 by condensation.

~~traffica con la carovana~~ ~~due~~ ~~collegio~~ ~~in~~ ~~la~~ ~~domenica~~ ~~...~~

5 sett. 77. Tris p. H.C. }  
30 sett. 77. ~~Tris~~ ~~Tris~~ ~~Tris~~ } p. C. W. S. M. M.

more 6.5.78



addition ~~the~~ test over 2 weeks in serum & 1.5  
over 2, when ran 5 points, a better test in 5.2a & 2.

5. iron ... smaller ... but it is much more flexible.

| Seq #          | 1.2     | Substrate | 1.2 |
|----------------|---------|-----------|-----|
| P <sub>1</sub> | 0.7     |           | 0.7 |
| B <sub>1</sub> | 7(5.0-) |           | 5.0 |
| B <sub>2</sub> | 7(5.0-) |           | 5.0 |
| C <sub>1</sub> | 0.7     |           | 0.7 |
| C <sub>2</sub> | 0.7     |           | 0.7 |
| D <sub>1</sub> | 0.7     |           | 0.7 |
| D <sub>2</sub> | 0.7     |           | 0.7 |
| E <sub>1</sub> | 7(5.0-) |           | 5.0 |

Conchocarpus  
*Conchocarpus* per m. jul. 10<sup>th</sup> - 1 m. a 500 m. a  
 6<sub>1</sub>, D<sub>1</sub> 1000 m. a 500 m. a 500 m. a 500 m. a  
 6<sub>2</sub>, D<sub>2</sub> 1000 m. a 500 m. a 500 m. a 500 m. a

Welllicht 2 P. *fructuosa* by clow 3  
(constituting 1st Div A, 1st clow 7 (1st & 2nd)  
above 2nd A, 1st clow. 1st & 2nd 12. In 1st clow 12

*[faint handwritten notes]*

problem text  
style text and LK?

more to be done absolutely  
 even to the same D.

Montevideo of J. no A. and J. no C. from book /

...  $10^2, 10^3, 10^4, 10^5, \dots$  ...  
 ...  $10^6$  ...

beantworte = 10<sup>15</sup> in 2. St. unverständlich mit dieselbe noch  
variablen bis 1/2

- Der A. greift auf voll. beschleunigung op. 1/20  
(beiden antennen)  $\gg$   $\ll$  1/20 nachschauen:  
nachlandseite

Given  $114^\circ$  true mag. at  $10^5$  ft. on horizontal line  
 but corrected dist.  $110^\circ$  mag.  $100000$  ft.  
 0.1 units. horizontal  
 then the underlining & with small mag. under point  
 the work done.

$\rho_{\text{max}} \approx 0.001 \text{ g/cm}^3$  bei  $10^{-7}$   
 $\rho_{\text{max}} \approx 0.001 \text{ g/cm}^3$  bei  $10^{-8}$

Many people like to have their hair cut

S. 100 A, as S. 100 C, ...  
 longhorn = backshell long horn 0.5 pup / pl over 1.0  
 smallhorn = stopper with longhorn in between  
 (backshell over horn mouth)  
 → collecting a metamorphosis  
 T. 2.

Wachstumsf., entwicklung., Kontrolle, ...

See A, p 71. - *unpublished manuscript*

6-2-52

22, 23

Mass  $\frac{1}{2}$  g. 2.5% w/v in 10% w/v solution.

Wie viel kann man abkassieren?

See A. <sup>6</sup> *Rhine* meting at imp. 2.0.5 lat c.p. to be considered.  
then, mean of given van der Bovenhaanheid

Montrer la poly I : C . ou  $p^{\infty}$  effectue une

F.I.F. : OK -  $\mu^m$  van merende rekening

W. A. Hartenstein  
(Boston machine)

7

612

240

©

100

50

up J.C. Clinton Nov. 2, 1891  
Syracuse  
(and 20 per cent)

bedrinking and eat the RWA  
most but with green sand.  
either on suspension.  
to other person, and  
with green bottle.

Das entspricht pa. ds. 2072

①  
Son in [unclear] - [unclear] [unclear] [unclear]  
4th [unclear] [unclear] [unclear] [unclear]  
[unclear] [unclear] [unclear]  
[unclear] [unclear] m. Son

Cell 128 Transloger Sim Transloger /  
 cellen is 5-10' /mole  
 procedure: concentration 2mL 37°C and with F.F.P  
 - Temperature 37°C  
 - (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> / 15% mol / 1 sample redolence  
 - diolysis 1.00 EXTRA 93% F.C.S.  
 - pH 2 - 2 HCl 100 pH 2.00 / 10°C / 1 sample  
 100%  
P. 128 in 6.1.2.2

■ PR52-9...

→ 125 mL + 100 µL F.F.P 10<sup>3</sup> L /mole redolence 4 mL 5.00  
 0.9

→ 125 mL + 100 µL F.F.P 10<sup>3</sup> L /mole redolence 4 mL 5.00  
 0.9

→ 125 mL + 100 µL F.F.P 10<sup>3</sup> L /mole redolence 4 mL 5.00  
 0.9

\* 0.2 - 0.1 mL also decontaminant

0.2 - 100 µL + A.B. 200 µL

0.2 - 100 µL + temperature 100 µL / 100 µL F.C.

0.2 - 100 µL +

temperature 100 µL (T.2)

0.2 - 2 mL + 100 µL decontaminant (100 µL 100 µL)

0.2 - 2 mL + (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> /

0.2 - 0.1 mL pH 2

0.2 - 100 µL 37°C

→ 125 mL (transloger) + F.F.P / 5.00 4 mL

\* 0.2 - 0.1 mL also decontaminant

0.2 - 100 µL + 100 µL F.F.P 10<sup>3</sup> L F.C.

0.2 - 100 µL + 100 µL F.F.P 10<sup>3</sup> L F.C. + 200 µL A.B.

■ clone 2 125 mL 5.00

0.2 - 0.1 mL also decontaminant

\* 0.2 - 100 µL + A.B. 0.2 mL

0.2 - 100 µL + A.B. 200 µL

0.2 - 100 µL + A.B. 200 µL

■ Control

→ 125 mL + 100 µL F.F.P 10<sup>3</sup> L F.C. = EXTRA 93% F.C.

0.2 - F.F.P 10<sup>3</sup> L F.C. = EXTRA 93% F.C.



Chart 3  
 1.1 ml 0.01% APDC by de Lysa 1 ml 5.00

Chart 4 1.1 ml 5.00

Co2 - also de Lysa  
 Co2 - 0.2 ml pbl  
 Co2 - 1.0 ml 16.5°C

inhibitor  
 2.7 0.2 ml buffer + FIE 10% E.C. pH 2  
 0.7 1.0 ml " " " 16.5°C

inhibitor  
 Co2 - 1.0 ml + 1.0 ml front stream  
 0.7 - 1.0 ml + 0.2 ml A.B. (apb)  
 0.2 - " " 2 ml A.B. (apb)  
 0.2 - " " 2 ml A.B. (apb)  
 0.2 - " " 2 ml A.B. (apb)  
 0.2 - " " 2 ml A.B. (apb)

trypanine inhibition  
 Co2 - 1.0 ml + trypanine 0.1 mg/ml 16.5°C  
 Co2 - " " " 2  
 Co2 - " " " 10  
 Co2 - " " " 50  
 Co2 - " " " 100

Co2 - 1.0 ml + F.I.E. (trypanine inhibition)  
 Co2 - " " trypanine 0.1 mg/ml 16.5°C  
 Co2 - " " " 2  
 Co2 - " " " 10  
 Co2 - " " " 50  
 Co2 - " " " 100

0.2 ml recommended by (no formal 4.0 ml)  
 0.7 ml (10% / 10% ↓)

1.0 ml buffer 0.1 mg/ml 16.5°C/ml  
 Co2

Station and off. from recommended on 10.00.

Behind: very exp. as next to be better  
 understand sensitivity to anti-fertilizer  
 E.C. needs adjustment to 6.6 pH 2.7. !



- 2.5, 2.5, and 2.5 ————— 8 and 5000
- 2.5, 2.5, and 2.5 ————— " " " " " "
- (1.5, 1.5, and 1.5) (1.5, 1.5, and 1.5) ————— " " " "
- (1.5, 1.5, and 1.5) (1.5, 1.5, and 1.5) ————— " " " "

43 *Acrotaphus abietalis* (V. & A. 1890) = 0 by hand-picking  
 10-11 sample near (V. & A. 1890) ...  
 p.s.k. each mean test at ... by hand-picking - p.s.  
 ... distance ...

(b) + tiny pin - no sound heard  
 + low area - 1.5% increase in volume  
 + water - 2.5% - no sound heard

heartless: poly I: C / pol. inhibited.  
 non structural poly II: C " " = same pol. inhibiting  
 as RNT

down. 7th wood ~~the~~ perambled down close, well  
 under the hostile  
 for the R. M. and  
 on the left. It  
 to make the point visible  
 with!



| Station | Time | Lat       | Long       | Alt | Wind | Temp | Humid | Clouds | Remarks      |
|---------|------|-----------|------------|-----|------|------|-------|--------|--------------|
| 1       | 0800 | 10° 15' N | 155° 00' W | 100 | 050  | 25.0 | 85    | 10     | Light breeze |
| 2       | 0900 | 10° 30' N | 155° 15' W | 110 | 050  | 25.5 | 85    | 10     | Light breeze |
| 3       | 1000 | 10° 45' N | 155° 30' W | 120 | 050  | 26.0 | 85    | 10     | Light breeze |
| 4       | 1100 | 11° 00' N | 155° 45' W | 130 | 050  | 26.5 | 85    | 10     | Light breeze |
| 5       | 1200 | 11° 15' N | 156° 00' W | 140 | 050  | 27.0 | 85    | 10     | Light breeze |
| 6       | 1300 | 11° 30' N | 156° 15' W | 150 | 050  | 27.5 | 85    | 10     | Light breeze |
| 7       | 1400 | 11° 45' N | 156° 30' W | 160 | 050  | 28.0 | 85    | 10     | Light breeze |
| 8       | 1500 | 12° 00' N | 156° 45' W | 170 | 050  | 28.5 | 85    | 10     | Light breeze |
| 9       | 1600 | 12° 15' N | 157° 00' W | 180 | 050  | 29.0 | 85    | 10     | Light breeze |
| 10      | 1700 | 12° 30' N | 157° 15' W | 190 | 050  | 29.5 | 85    | 10     | Light breeze |
| 11      | 1800 | 12° 45' N | 157° 30' W | 200 | 050  | 30.0 | 85    | 10     | Light breeze |
| 12      | 1900 | 13° 00' N | 157° 45' W | 210 | 050  | 30.5 | 85    | 10     | Light breeze |
| 13      | 2000 | 13° 15' N | 158° 00' W | 220 | 050  | 31.0 | 85    | 10     | Light breeze |
| 14      | 2100 | 13° 30' N | 158° 15' W | 230 | 050  | 31.5 | 85    | 10     | Light breeze |
| 15      | 2200 | 13° 45' N | 158° 30' W | 240 | 050  | 32.0 | 85    | 10     | Light breeze |
| 16      | 2300 | 14° 00' N | 158° 45' W | 250 | 050  | 32.5 | 85    | 10     | Light breeze |
| 17      | 2400 | 14° 15' N | 159° 00' W | 260 | 050  | 33.0 | 85    | 10     | Light breeze |
| 18      | 2500 | 14° 30' N | 159° 15' W | 270 | 050  | 33.5 | 85    | 10     | Light breeze |
| 19      | 2600 | 14° 45' N | 159° 30' W | 280 | 050  | 34.0 | 85    | 10     | Light breeze |
| 20      | 2700 | 15° 00' N | 159° 45' W | 290 | 050  | 34.5 | 85    | 10     | Light breeze |

033. comitatus hanc hanc. nuplex hanc  
 pamplos nunc hanc  
 comitatus hanc

ASC . . . . .  
aufgekl. . . . .  
19 12.4 g. propan

down - around two in tent, when taken better by 0.5  
 given by 10th the next morning when tent

... ~~... ..~~ ... on E. 517. 21.8

<sup>1</sup> = carbon no. on the chain.

Shore. vegetation where lowest reaches 100 ft. about 100 ft.  
about 100 ft. (normal then to 100 ft. E. 100 ft.)  
100 ft. S.E.

[illegible]



10115179

OT

deurlopen. licht. verkleuring

opmerkelijk (van schand)

pH 2.5 analyse per uitsluiting 0.5 - 0.5  
Rivier per uitsluiting 0.5 - 0.5

10115179

OT

(van schand)

pH 2.5 analyse per uitsluiting 0.5 - 0.5  
Rivier 0.5 - 0.5

aan de hand 0.5

10115179

Deurlopen. licht. verkleuring

10115179. 10115179. 10115179.

10115179. 10115179. 10115179.

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10115179. 10115179. 10115179.

10115179

aan de hand 0.5

10115179.

10115179. 10115179. 10115179.

10115179. 10115179. 10115179.

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aan de hand 0.5

10115179. 10115179. 10115179.

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10115179. 10115179. 10115179.

10115179. 10115179. 10115179.



On 3-1-74 the following

101515-5 @ 1.5 of T2

Station 900 - 1000 ft

- moderate wet soil  
 my. moderate by 1.5%  
 per 10.5

- 10% reduction in 1.5%  
 1.5% wet soil 1.5%  
 1.5% wet soil 1.5%  
 1.5% wet soil 1.5%

mean 1.5%  
 1.5% (1.5% - 1.5%) wet soil 1.5%

- 10% reduction in 1.5%  
 1.5% wet soil 1.5%  
 (mean of 1.5% - 1.5%)  
 1.5% wet soil 1.5%

mean 1.5%  
 1.5% (1.5% - 1.5%) wet soil 1.5%

- 10% reduction in 1.5%  
 (mean of 1.5% - 1.5%)

of T2: 1.5%  
 FRK: 1.5%  
 E, 1.5% 1.5% wet soil 1.5%

101515-5

@ moderate wet soil  
 1.5% wet soil 1.5%

- 10% reduction in 1.5%

- 10% reduction in 1.5%  
 (mean of 1.5% - 1.5%)  
 1.5% wet soil 1.5%

- 10% reduction in 1.5%

@ moderate wet soil

per 1.5% wet soil 1.5%

1.5% wet soil 1.5%

sep 130      ~~Handwritten~~ ~~Sept~~ ~~130~~ ~~130~~  
 ch 3 3-3      ~~Handwritten~~ ~~Sept~~ ~~130~~ ~~130~~  
 ch 7      ~~Handwritten~~ ~~Sept~~ ~~130~~ ~~130~~  
 ch 3      ~~Handwritten~~ ~~Sept~~ ~~130~~ ~~130~~  
 ch 7      ~~Handwritten~~ ~~Sept~~ ~~130~~ ~~130~~  
 of 2-5 A - ~~Handwritten~~ ~~Sept~~ ~~130~~ ~~130~~

ch 7      ~~Handwritten~~ ~~Sept~~ ~~130~~ ~~130~~  
 3-3      ~~Handwritten~~ ~~Sept~~ ~~130~~ ~~130~~  
 3-3      ~~Handwritten~~ ~~Sept~~ ~~130~~ ~~130~~  
 3-3      ~~Handwritten~~ ~~Sept~~ ~~130~~ ~~130~~  
 3-3      ~~Handwritten~~ ~~Sept~~ ~~130~~ ~~130~~

a) ch 3 - ~~Handwritten~~ ~~Sept~~ ~~130~~ ~~130~~  
 ch 3-3      ~~Handwritten~~ ~~Sept~~ ~~130~~ ~~130~~  
 ch 3-3      ~~Handwritten~~ ~~Sept~~ ~~130~~ ~~130~~



Let's E-DC ground up over much  
 volume

--- Ten 2-4-50

Franklin in paragraph

T-1

G-307

Conditions

Salmonella subunit

may be affected by way 1 and

R-15

0.3"

0.6

2.5 F

10<sup>2.2</sup>  
 10<sup>2.2</sup> by

10<sup>2.2</sup>  
 10<sup>2.2</sup> by

poly 2 C

0.007 inch

0.007 inch

fr-4-4-50

0.07 inch

0.07 inch

Handwritten: Let's see - I.C. - 100 is,  
then because in the 100% but  
... ..  
... ..  
... .. in negative ...



East L.C. in. of 2.5th and 3rd. (E. 10)

|      | London Bond St. | New York Bond St. |
|------|-----------------|-------------------|
| 1000 | 1000            | 1000              |
| 1000 | 1000            | 1000              |
| 1000 | 1000            | 1000              |

dem Kampf bis 1917. Die Aktivität ~~unverändert~~  
 1917-1918. war sehr auf. war streng mit dem Am 1. 1918

sch 6 - ... ..  
... ..  
... ..  
... ..  
... ..  
... ..

Das Enzym *exp.*  
 En is bekend dat als onderzocht  
 ———— *pt. 1-9*  
 En een bekend *exp.* gemaakt worden dat  
 het synthetisch is.

Excluded part to basin of Little Lake - of E. Long

~~\_\_\_\_\_~~  
Two 5 km down down

*Handwritten signature*

- p 7659 - Ectophasma paucithorax

Lander Pm B. m. Bm B.,  
exp r rr a  
dian ab tr tent blight

*✓ Not for - even under*

*understand*

*below is*

*nothing?*

